

Europe's HIV-contaminated blood

The origin of the blood and blood-products contaminated with HIV sold on the European market is more probably a mark of incompetence than of cupidity, but is none the less culpable on that account.

THE alarm that spread through Germany and much of the rest of Europe last week about the supply of potentially contaminated transfusion blood and other blood products raises awkward questions about the safety of the public health in circumstances in which biological materials of various kinds are traded internationally. The late Richard Titmuss, the professor at the London School of Economics who argued eloquently (in a book called *The Price of Blood*) that only freely volunteered donations can be trusted, appears to have been thoroughly vindicated. Although the company now recognized as the source of contaminated products in Germany and elsewhere in Europe appears routinely to have tested blood donations for HIV (among other contaminants), it did so in a way that could only have reduced the sensitivity of the assay. Like any other commercial organization, it was seeking to save time and trouble, and thus money. Titmuss's argument was that this is only to be expected.

The real trouble goes deeper, but it is not irrelevant that the French scandal over blood transfusions contaminated with HIV happened soon after the National Transfusion Service had been made commercially free-standing and instructed to balance its books by offsetting the costs of its domestic operations with the surpluses to be made from its monopoly on imported blood. That was the impetus for its director's infamous instruction that contaminated blood should continue to be supplied "while stocks last". But Michel Garretta is in jail, as is his once-colleague Jean-Pierre Allain on the flimsy charge that his warnings of disaster were not public enough. So does it not follow that the managers of the German company responsible for supplying contaminated blood should also end up in jail?

The difficulty is that the fault in Germany is more probably attributable to incompetence than to cupidity. If, as reported, a test for HIV was applied to pooled blood rather than to single donations separately, the person who devised that procedure must have been ignorant of the inevitable finite sensitivity of such tests. Otherwise, commercial considerations would have argued against cutting corners; when so much is known of the risks of transfusing HIV-contaminated blood, it would have been apparent that the cost of being found in the wrong must far exceed whatever might be saved by reducing the numbers of blood samples tested.

That is why the essence of the German case is whether incompetence is a crime and, if so, what kind of crime. As so often happens, the question is not novel, but its setting is. For is it not part of the tradition of professions such as engineer-

ing that people who give faulty advice in the construction of, say, a bridge are held culpable if, afterwards, the construction fails? More recently, accountants who are found to have audited incompetently the accounts of companies for which they are responsible are similarly liable to lawsuits from those injured in the process. Then motor-car manufacturers supplying dangerous products must expect to be sued for whatever damage is done to those who acquire them. Those who allow contaminated blood onto the market cannot therefore expect to be exempt from whatever penalties are prescribed for incompetence and carelessness.

But none of that will help when the consequences of incompetence may be as scary as they must be when even small amounts of contaminated blood and other biological products find their way into general use. Titmuss's argument that once there is a market in such materials the quality of the materials supplied is inherently untrustworthy has force, but is impractical. Even the best-run public health systems are under pressure to supply themselves with all the biological materials they could use. So the trick is not to do without a market, but to operate in such a way as to avoid the pitfalls of which Titmuss warned. And that can be done only if those who buy products in bulk from the market undertake their own testing of what they have paid for.

But double-checking along these lines cannot always be effective. The use of pituitary glands as a source of human growth hormone has, in the past decade, been recognized for the first time to be a possible agent of Creutzfeldt-Jakob disease in a number of patients, but even now there is no assay of the infectious entity. What the law appears to hold in such cases is that there is a distinction between involuntary and voluntary ignorance. When there are no means by which a person can know that some material is dangerous, he is not guilty of incompetence if later developments prove his confidence misplaced. But that does not apply to incompetence in a simple matter of measurement. □

An end to fraud?

The responsibilities of the US Office of Research Integrity should be returned to the research community.

THE decision last week of the Departmental Appeals Board of the US Department of Health and Human Services (see page 99) is welcome on two grounds: it exonerates an honest



scientist, Mikulas Popovic, of charges of misconduct levelled at him by the department's own Office of Research Integrity (ORI) and it throws into question the value and role of ORI itself. Or, rather, it demonstrates to be mistaken the ambition of the US National Institutes of Health (NIH) to create a novel quasijudicial process to determine allegations of scientific misconduct. NIH first created its Office of Scientific Integrity (OSI) in the hope of resolving serious allegations against researchers without formality; its officials used to claim that they would resolve even contentious cases much as researchers tackle problems in science, by investigation and inference. The first admission that OSI was out of its depth came two years ago, when it was transmogrified into ORI. Last week's decision could be the last nail in its coffin.

OSI has always been the wrong way to deal with questions of misconduct. Its inspiration appears to have been NIH's central but not exclusive role in the pattern of US research and its awareness of its own power, notably that of denying further research support to people in its black books. To be fair, NIH has also been under pressure from the US Congress to ensure that public funds used to support research are honestly spent, although it might have done more than it has to persuade congressmen that cases of scientific misconduct differ qualitatively from the financial scams with which they are more familiar. Even so, NIH should not have allowed itself to be trapped into the implicit promise that it would police the research it supports, and enforce administrative penalties against those found guilty of misconduct, without running into legal difficulties.

That is how it has now been tripped up. To declare that a person is no longer eligible for research support amounts to deciding that his or her career as a scientist is at an end. For a public institution such as NIH to make such a decision publicly is unthinkable without the paraphernalia of what the constitution calls "due process" — the right to representation by counsel, the right to hear the allegations and to question those who make them, seemingly rules on the admissibility of evidence and so on. But OSI did not follow these rules, nor pretend to. Nobody should be surprised that it has now been found deficient. But there is no chance that OSI's successor, ORI, can be reformed to meet the public need that justice should not only be done but should be seen to be done. Better to abolish ORI.

That does not mean that NIH can wash its hands of misconduct, or even that would-be fraudsters can falsify data freely. On the contrary, the demise of ORI could pave the way for more effective mechanisms. As the research world knows, misconduct is best dealt with at the institutional level, where close colleagues (and whistle-blowers, if there are such) can confront those acting suspiciously. But experience shows that institutions prefer to hide from unpalatable truths. NIH could help to ensure that this does not happen at institutions where its own funds are engaged by insisting on investigations by truly independent committees and by making the findings public. Other public agencies, of course, should follow suit. Meanwhile, NIH should firmly explain to the Congress that there are unavoidable limits to

its power to act as policeman even for biomedical research in the United States.

Suspensions that the result would be that people given to mishandling data would then be free to falsify at will are entirely misplaced. Just as close colleagues are often the best judges of the integrity of a person's work, so by their decisions on matters such as collaboration and promotion are they often the most effective arbiters of his or her continued success. It will be excellent if responsibility for deciding on misconduct cases can be returned to where it belongs, the research community, and if the community can be kept up to the mark in vigilance and fairness. The tragic interruption of Dr David Baltimore's career should show that the penalties can be severe enough to satisfy the most bloodthirsty congressman. □

A date with Darwin

Britain is getting good value for money from its overseas biodiversity programme.

MORE than a year after the surprise commitment by the British prime minister, John Major, to underwrite research into conservation biology, made in June 1992 at the UN Conference on Environment and Development in Rio de Janeiro, a little money is to be spent. Some may say that the money — £6 million over three years — is too little too late. But in the Darwin Initiative, the perennially unpopular Mr Major may at last have done something right, for the plan is a genuinely good idea in which British expertise could have a distinctive part to play.

Six million pounds is of course a paltry sum, but the intention of the Darwin Initiative was never to appropriate large sums for big projects in which other bodies are already engaged. Rather, it was meant as a resource to seed the activities of smaller organizations with grants of a few tens of thousands of pounds. The emphasis on the training of researchers in developing countries is especially sensible; that should ensure that what funds there are will be used efficiently. Britain may yet find itself applauded despite the small price it has paid for the grand promises made in Rio.

This year's grants awarded from this small pot (see page 100) may even help to quieten past passions over systematic biology in Britain. After all, it is only three years since the Natural History Museum's 'corporate plan' caused such a stir that the House of Lords was moved to a formal inquiry into the perilous condition of the field. In passing, the Darwin Initiative may help to fill some of the gaps then identified. It will also reinforce the impression that has been growing over the past few months that clever marketing by the Natural History Museum, the London Zoo (now not endangered) and the Royal Botanic Gardens at Kew, which together epitomize biodiversity in the public mind, has transformed biodiversity from the pursuit of bearded visionaries to the legitimate — even fashionable — concern of millions. Major may one day be asking how many of them vote Conservative. □

Recession forces R&D cuts in Japanese industry

Tokyo. The prolonged recession is biting hard into the research and development budgets of Japan's major electronics companies. As a result, many companies are changing the way they organize their research in order to make it more productive and cost-effective. If the recession continues, the future of the basic research they support may be threatened.

A survey of 10 leading electronics and telecommunications companies, which between them account for more than a quarter of Japan's industrial research and development, shows that ¥131 billion (\$1.24 billion) has been cut from the peak of ¥2,801 billion (\$26 billion) spent in 1991.

Hitachi, the biggest spender, has reduced its research spending by ¥40 billion and Fujitsu by ¥62 billion. Only the telecommunications company Nippon Telegraph and Telephone (NTT) predicts a significant increase in budget, after holding research and development spending flat in 1992.

Such companies had been hoping that Japan would be pulling out of recession by now. But an unusually cool summer resulted in reduced demand for products such as air conditioners, while the rising value of the yen has cut the value of overseas sales.

At present, there is no sign of an improvement in their fortunes, despite pump-priming measures by the government. As a result, forecasts for research and development spending in the current fiscal year, which runs to the end of next March, have been revised downwards.

The cutbacks in spending inevitably have caused difficulties. But they have also pro-

vided an opportunity for managers to introduce new methods of organization that have been planned for a long time.

Researchers at Sony, for example, are being asked to raise a significant portion of their research funds from the 24 business groups that make up the company, rather than from corporate headquarters. This is making their research more product- and goal-oriented (*Nature* 361, 193; 1993). And last month, NEC introduced a scheme — revolutionary by Japanese standards — that rewards the achievements of individual researchers.

Under NEC's "quality work" scheme, the most senior unionized researchers (*shunin*) in charge of small research groups are required to set their research goals. Their performance will be reviewed every six months, through both self-evaluation and an assessment by their colleagues and research managers. The output and quality of publications, as well as the number of patents filed, will also be taken into account.

The size of the researchers' six-monthly bonuses will be determined by their ranking on a scale of 1 to 10; the highest rank will get 30 per cent more than the lowest. The new salary package also includes a fixed payment for overtime regardless of hours spent, and more flexible working hours, including the right to work at home on research papers. Overall, the changes should save the company money, and if successful, they probably will be extended to other levels of researchers in the company.

Such practice runs counter to tradition in Japanese industry where everyone is treated equally and salary rises with seniority. But NEC research managers, who have been planning the introduction of this scheme for several years, are confident it will lead to better research performance, particularly in areas of more basic research.

Throughout the 1980s, spending on research and development by Japanese industry rose steadily, particularly during the economic boom at the end of the decade, when companies poured money into the basic research institutes and laboratories were set up to carry out blue sky research. The spending spree hit a peak in 1991 and for many companies investment in research has dropped sharply since then.

David Swinbanks

Australian minister clashes with CSIRO on marine research

Sydney. Australia's Minister for Science and Small Business, Chris Schacht, seems set to clash with government scientists over his plans for the reorganization of public research. Last week Schacht revived a proposal, previously shelved, to shift two sections of the country's major research body, the Commonwealth Scientific and Industrial Research Organisation (CSIRO), into a



Schacht

separate marine research institute.

Schacht announced the move after he had received an independent report on the initial proposal. So far, CSIRO has not responded to the new announcement. But both its

officials and scientists are believed to be just as opposed to the idea as when it was first put forward in July.

Schacht originally proposed forming a giant National Institute of Marine Science by amalgamating the existing Australian Institute of Marine Science (AIMS) with the CSIRO divisions of fisheries, oceanography and atmospheric research.

But the outcry from both the CSIRO and sections of the scientific community was sufficient to make the government call for a report on the proposal from Ken McKinnon, vice-chancellor of the University of Wollongong, and the author of earlier studies of marine policy.

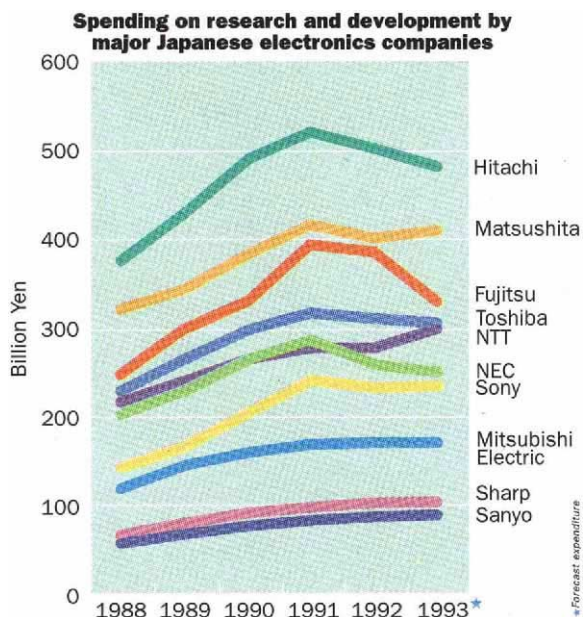
McKinnon's report, delivered last week, provides ammunition to both sides. On the one hand, it says that an oceans management policy is "essential to the national interest", and that "present organizational arrangements fall well short of what is required".

But McKinnon also comments that, while the amalgamation proposal had "attractive features", it is still flawed, as the proposed institute would not oversee all of the government's marine research effort.

One solution, says McKinnon, would be to include all other government bodies involved in marine research, for example the Great Barrier Reef Marine Park Authority. But before then, the government should first set up an Australian Marine Industries Sciences Council (AMISC), he says.

Both Schacht and CSIRO have welcomed McKinnon's proposal. But Schacht also issued a press release stating that the review had effectively endorsed his own proposals for the restructuring of science agencies in his portfolio, a move which has done little to improve his relations with CSIRO.

Mark Lawson



Creutzfeldt-Jakob verdict may prompt new claims

London. In what may be only the first in a succession of similar cases, a verdict of "medical misadventure" was reached last week in Lincoln following an inquest into the case of a young man who died of Creutzfeldt-Jakob disease (CJD), contracted as a result of treatment with contaminated human growth hormone.

CJD is a neurodegenerative disease characterized by rapidly developing dementia; death follows often less than a year after onset. It is related to bovine spongiform encephalopathy (BSE), better known as 'mad cow disease', and the sheep illness scrapie.

Although familial clusters of inherited CJD exist, overall incidence in the general population is approximately 1 in 10,000 individuals, with an average age of onset of 61 years. For this reason, the deaths of three young adults from CJD reported in early 1985 in the United States were suspicious.

The three were among 7,000 people who had been treated with pituitary growth hormone derived from cadavers, a practice carried out in many countries to treat growth deficiencies. Such use of the hormone was suspended in the United States, and the United Kingdom followed in May the same year, after a confirmed case of CJD. Both countries authorized use of recombinant human growth hormone later that year.

CJD can be transmitted by infection with particles containing an abnormal form of the prion protein (see *Nature* 365, 386; 1993). The brains of CJD-infected cadavers contain these infectious particles, which are highly resistant to most common sterilization procedures.

Cadavers of affected individuals not diagnosed for CJD could contain prion particles without the brain having the spongiform appearance typical of late CJD. As preparation of one batch of hormone required up to 20,000 pituitary glands, it was not unlikely that a batch could

have become contaminated.

The case considered by the inquest jury is certainly not isolated, as ten of the 1,900 patients treated with growth hormone in Britain between 1958 and 1985 have already died. Families of those treated with growth hormone have asked the British government to agree to pay compensation similar to that given to haemophiliacs infected with HIV by contaminated blood products. But the government is denying liability, on the grounds that precautions were taken as soon as the risk became evident.

The problem of contaminated growth hormone is not limited to Britain. Legal procedures have been opened against two French doctors, charging them with "involuntary homicide" in supplying contaminated hormone (see *Nature* 364, 372; 1993). Cadaver-derived hormone was used in France until the end of 1988, when the recombinant product was introduced.

The French government has now announced that, whether or not any negligence was involved, it is prepared to assume full responsibility, and that people with CJD will be compensated. Legal procedures already under way will not be interrupted. But legal action against those responsible for possible malpractice is a separate issue from compensation for the victims.

There is still a large number of people worldwide who were treated with possibly contaminated products but who have not developed CJD. The incubation period can be up to 40 years, but as recent studies indicate that there is a genetic predisposition for iatrogenic CJD, owing to contaminated growth hormone or contaminated dura mater used in brain surgery, it may be possible to identify those at greater risk.

Meanwhile, the question of who is responsible when such contaminations occur rages on in both Italy and Germany (see right) where scandals over contaminated blood are just breaking.

Kimberly Carr

Concern grows over impact of German blood scandal

Munich. The German AIDS scare, which began with the closure of the pharmaceutical blood product company UB Plasma in Koblenz, developed this week into a nationwide panic after Horst Seehofer, the health minister, recommended on 3 November that anyone who received blood or blood products since the early 1980s should undergo an HIV test.

UB Plasma was accused two weeks ago of failing to screen all its blood products for HIV contamination. Four employees have so far been arrested. Products have been distributed widely throughout Germany and other European countries. In addition, 500 litres of untested blood from Romania, delivered to UB Plasma but stored at its Vienna-based partner company, Octaplasma, have disappeared; it is not known if the blood has been sold to hospitals.

Seehofer has been criticized for creating a panic that has caused thousands of individuals to back out of both routine and in some cases urgent surgery. Doctors have warned that refusing treatment can present a higher risk to patients than that of receiving infected blood products, and that Germany may lack the capacity to carry out all the tests likely to be demanded.

So far, only three cases of AIDS infection have been associated with UB Plasma products. But more could appear. The regional state government of Rhineland-Pfalz is to test samples from each of the UB Plasma's blood donors, of which there are estimated to be about 25,000, in order to pinpoint the exact source of infection.

Seehofer is disbanding the federal health bureau which, he said, had been careless about reporting cases of AIDS infection which had been caused by blood transfusion (see *Nature* 365, 687; 1993). But carelessness may have been even more pervasive. The news magazine *Der Spiegel* this week reported that the district council of Koblenz had been told of suspicions about UB Plasma's practices as long ago as 1987, but had filed the report without acting on it. Last week, the director of pharmaceuticals in the district council, Gerhard Frick, was relieved of his duties relating to control of blood and blood products.

Der Spiegel believes that a much wider conspiracy exists; it also reports that a consultant at the Bonn University Clinic, the biggest haemophiliac centre in the world, has had more than DM2 million transferred into a numbered Swiss bank account.

Officials at federal and local levels are trying hard to contain the crisis, recalling UB Plasma products, choosing safe replacements for them and offering free, anonymous AIDS testing.

Alison Abbott

If Paracelsus had been born in 1993 instead of 1493, his leanings towards mysticism could have lead him to a life in modern Europe's alternative circles. But Paracelsus — born Philippus Aureolus Theophrastus Bombastus von Hohenheim — was even more of a thorn in the flesh of the establishment, challenging accepted medical views to the point of publicly burning medical textbooks while teaching at the University of Basel.

He was forced to flee academia in 1528 under threat of arrest, and spent the rest of his life wandering among the peasants of central Europe. Responsible for more than 200 publications covering aspects as diverse as folk medicine, alchemy and cosmology, Paracelsus is now widely considered the founder of medical chemistry. Five hundred years after his birth in Einsiedeln Switzerland, a celebratory stamp was issued in Germany this week to pay homage to this scientific debt. Last year he was rehabilitated by the Pope.

A. A.



Varmus deft with Senate on NIH strategy

Washington. Harold Varmus, director-designate of the US National Institutes of Health (NIH), appeared before the Senate last week for confirmation hearings that, while calm on the surface, nonetheless revealed an undertow of potential conflict.

Members of the Senate Committee on Labor and Human Resources, chaired by Edward Kennedy (Democrat, Massachusetts), took turns praising Varmus as a literary scholar (he has a deep interest in seventeenth-century literature) and as a star scientist who would be NIH's first director with a Nobel prize. Varmus and Michael Bishop shared the 1989 prize for physiology or medicine for their discovery that viral cancers derive from cellular genes or oncogenes.

But a barrage of questions from the committee made it clear that Varmus will have no easier a time than his predecessors in dealing with Congress's long-standing habit

of trying to determine the details of the NIH's research portfolio by focusing on diseases of special interest to individual members and their constituents.



Varmus: will need 'a lot of steel'.

Only minutes into the hearing, Christopher Dodd (Democrat, Connecticut), admitting that he might be unfairly raising a subject beyond Varmus's knowledge, repeated the story of a little girl with an unusual urea cycle disease that is being treated at NIH. Only 124 people have the disease, and the US Food and Drug Administration has yet to approve the experimental drug

used for its therapy. Varmus, well-briefed and thinking quickly, reassured Dodd that NIH would not abandon the urea cycle patients, however few they may be.

Barbara Mikulski (Democrat, Maryland), in whose state NIH is situated, instructed Varmus to pay attention to one of her favourite causes, women's health, and said she would visit him soon to talk about it. Varmus may well ask Ruth Kirschstein, former director of the National Institute for General Medical Science, NIH's most basic-research institute, and interim head of women's studies, to sit in. Varmus has already announced that Kirschstein will be the NIH's new deputy director.

Paul Wellstone (Democrat, Minnesota) raised the issue of Parkinson's disease. Both his mother and father have the disease, he told Varmus, adding that NIH was not doing enough for these patients.

Varmus replied by explaining that Parkinson's is a disease of the basal cell ganglia. Drawing on what is likely to be the underlying theme of his tenure at NIH, Varmus tried to convince Wellstone that much basic research — such as that in the neurosciences — is directed at diseases such as Parkinson's, even though it might not be labelled as such on the budget tables.

Wellstone, for his part, seemed politely unconvinced and said he would like to "visit with" Varmus about this again. But Varmus maintained his defence of basic research. "As an investigator who has seen the pursuit of an obscure chicken virus create a new vision of human cancer, I will defend open-ended basic science against the calls for restricted applications of what is already known," he said. Dan Coats (Republican, Indiana) told Varmus that, as leader of NIH, he will "need a lot of steel". Coats did not mean it as a joke.

Perhaps next time Varmus should borrow a trick from Ross Perot, and come armed with a set of eye-catching charts to show the links between basic science and the diseases that dominate the congressional and public mind.

Barbara J. Culliton

Popovic wins appeal against ORI charges

Washington. Mikulas Popovic, the former US National Institutes of Health (NIH) researcher who succeeded in 1983 and 1984 in growing the human immunodeficiency virus (HIV) in sufficient quantities to develop a blood test, has been cleared of all charges of scientific misconduct in writing up the paper in which he reported his data.

After years of investigation into questions about the integrity of certain words and figures in the 1984 paper, coauthored with his laboratory chief Robert C. Gallo and published in *Science*, NIH found Popovic, who worked at the National Cancer Institute at the time, guilty of misleading readers on four counts.

In one, for instance, the NIH's Office of Scientific Integrity (OSI), found him guilty of misconduct for using the initials ND (for 'not done') in a table in which the 'ND' experiments clearly had been done (even though it agreed that this in no way altered the scientific validity of the paper).

The OSI, and then its supervisory (and now successor) agency, the Office of Research Integrity (ORI), rejected Popovic's argument that he meant ND to mean 'not determined', as well as claims that ND is used in the literature with both meanings. Popovic appealed his conviction for misconduct on this and other similar counts to the Departmental Appeals Board within the Department of Health and Human Services, the highest court of appeals within the government's internal disciplinary system.

The appeals board, whose members are all trained in law, held a 12-day hearing of the charges. On 3 November, it issued its judgement, saying: "One might anticipate that from all this evidence [presented by OSI and ORI], after all the sound and fury, there

would be at least a residue of palpable wrongdoing. That is not the case."

The board said that the OSI and the ORI, which had been both accusers and judges of Popovic, were bound by the normal legal standard that conviction requires proof of guilt by a "preponderance of the evidence", rather than by a pyramid of assumptions built on a foundation of inferences. "Specifically, ORI did not prove that the *Science* paper contains untrue statements or data, much less that it contains intentional falsifications," the board said in its 79-page opinion.

The board rebuts the ORI's case point-by-point and emphasizes that a finding of scientific misconduct, which stigmatizes researchers and can deprive them of their livelihood, must be based on legally supportable evidence. Popovic was unemployed for about three of the five years that his case has been making its way through the system.

ORI's director, Lyle Bivens, says that if his agency has to meet the appeals board's standards of proof and intent to deceive, it will have a hard time proving misconduct. The board's response is that that is as it should be, because the stakes for the accused are so high.

Unless ORI tries to interest the US Justice Department — where standards of guilt would be equally high — in filing criminal charges against Popovic, the board's decision represents the last hurdle for Popovic, who has run up legal fees of close to a quarter of a million dollars. He is now working in a laboratory at the Karolinska Institute in Stockholm, Sweden. But he says that, even after everything he has been through, he would still like to return to the NIH.

Barbara J. Culliton

Research funds grow

Capetown. Spending on research and development in South Africa went past the one per cent level of gross national product for the first time in 1991/2, according to the latest biennial survey, compiled for the first time by the Science Policy Unit of the Foundation for Research Development.

Expenditure, at 1.04 per cent of GDP, represents a significant increase since 1989/90, when it stood at 0.86 per cent. But William Blankley, a researcher at the unit, feels that the increase can be explained in part by the inclusion of more companies in the survey.

Michael Cherry

Royal Society urges rethink on degrees

London. British universities should abandon the traditional 'honours' system for classifying undergraduate degrees on a single scale of 'firsts' to 'thirds', and adopt the practice followed by the United States and many European countries, where a more comprehensive record of achievement is provided, according to a report published this week by the Royal Society.

The report also endorses an initiative being backed by the Institute of Physics through which some universities have introduced simplified undergraduate courses in science subjects, complemented by additional studies, leading to a four-year degree course, for those intending to become professional physicists.

The widespread adoption of four-year courses for science degrees has been opposed by the government on the grounds of cost. But the Royal Society suggests that the physicists' approach, which includes the possibility of "less able" students finishing their higher education after only two years, should be extended to other areas of science and mathematics.

"We have no doubt that an extended and enhanced degree is the minimum required to educate scientists and engineers to full professional levels comparable with those reached by their peers in other EC countries", says the report, which was prepared by a study group chaired by Sir Eric Ash, the former rector of Imperial College, London.

Its main recommendation is that higher education institutions need to be able to offer a range of qualifications, and must allow students to select those most appropriate

to their educational and employment needs. And it adds: "We believe that the time to bury the 'honours classification' has arrived."

Meanwhile, the Royal Society is one of a number of scientific organizations that have supported a proposal to establish a new body to oversee the development of vocational qualifications for British scientists, technologists and mathematicians.

The proposal has been made by a working group set up by the Council of Science and Technology Institutes (CSTI). Backed by the Department of Employment, its main goal would be to encourage courses to improve the professional skills of scientists and technologists.

The aim is to achieve this within the framework of the government's broader initiative of introducing National Vocational Qualifications (NVQs) across all sectors of British industry.

A survey conducted by the CSTI on behalf of the department and published earlier this year found that about 600,000 people are currently employed in Britain in jobs where science, technology and mathematics are considered to be the main function.

In addition, 1.7 million have jobs where knowledge of one of these fields is essential to their professional competence. And a further million are in positions where some such knowledge is felt to enhance their effectiveness.

Future employers of scientists are not the only supporters of the initiative. The Institute of Professionals, Managers and Specialists, the trade union representing many government scientists, says that it is keen to be represented on any new overseeing body to ensure that the needs of its members — as well as those of their employing organizations — are fully met by the new qualifications.

David Dickson

Darwin funds to back biodiversity

London. Twenty-six British and international projects aimed at conserving biodiversity are to be funded under the United Kingdom's Darwin Initiative. Initial grants totalling £3.28 million over three years, announced on 3 November by John Gummer, the environment secretary, are just over half the £6 million that the government has promised to spend on the scheme — announced by John Major, the Prime Minister, at last summer's UN Conference on Environment

and Development in Rio de Janeiro.

High priority was placed by the committee which advised the Department of Environment on the value of training conservation biologists in developing countries: training makes a little money go a long way. As a result, £1.53 million has been allocated to nine projects explicitly concerned with training.

The biggest beneficiaries are the World Wide Fund for Nature (two projects worth a total of £522,100), a project sponsored by the British Ecological Society (£356,000) and the International Mycological Institute (IMI, £305,300). "Training and education is one of the most effective ways of using a resource of this kind", says David Ingram of the Royal Botanic Gardens, Edinburgh, a sponsor of several bids to the advisory committee.

But the boundary between support for training and research is not rigid. David Hawksworth of the IMI says that its award will fund 22 'Darwin fellows' in several developing countries, concerned with research problems in parasitology, bacteriology and entomology, as well as mycology.

Of the £6 million, the advisory committee spends £1 million in the first year, with £2 million and £3 million in subsequent years, allowing for continuing commitments made in earlier years. This gives the advisory committee plenty of room to play with, says Sir Crispin Tickell, warden of Green College, Oxford, and chairman of the committee, who urges institutions with good ideas to send in applications. Further announcements are expected early next year, as applications are processed. **Henry Gee**

Future of Rutherford under debate

London. Britain's largest surviving public research laboratory, the Rutherford-Appleton Laboratory (RAL), will begin life under the new arrangements for supporting research as a dependant of the Engineering and Physical Sciences Research Council (EPSRC). But proposals for more radical reorganization are being drawn up and are due to be completed by the middle of this month, in time for a decision by next April, when the new system will come into effect.

RAL, now financed directly by the Science and Engineering Research Council (SERC), is due to be amalgamated with the Daresbury Laboratory. The government's white paper on research decreed that the two laboratories would be run directly by the Office of Science and Technology by its director-general of research councils, whose appointment is imminent.

The rationale of the combined laboratory is that of providing a service for academic research groups. Since the disbandment of its nuclear structure research group last year,

the Daresbury Laboratory's chief role is as Britain's synchrotron radiation laboratory, widely used by non-British research groups.

The Rutherford-Appleton Laboratory proper, 80 miles south of Daresbury, owes its existence to the merger in the 1970s of radio research and high-energy physics laboratories on a site adjoining the Harwell laboratory of the UK Atomic Energy Authority.

The amalgamated RAL will employ well over 1,500 qualified scientists. Its present anxiety is that there is no obvious place in what is intended to be a market-led research enterprise for a general service laboratory with its particular mixture of skills.

The laboratory's preferred option appears to be an arrangement under which the research councils would purchase a range of research services much like that now on offer. Other options, especially that in which research services would be purchased directly by those holding research grants, are less likely to be welcome. **John Maddox**

Dutch favour applied research for natural gas 'dividend'

Paris. Next year's Dutch research budget will impose yet more cuts on fundamental research, share out extra income from the country's natural gas resources mostly on applied research, and provide big new tax incentives to companies to do more research. These moves continue the trend of reduced overall spending on science and increased emphasis on wealth creation.

In 1987, the Netherlands spent 2.33 per cent of its gross national product on research. Now it spends 1.8 per cent. The enormous costs of its social welfare system and its small population of taxpayers has made Dutch research more vulnerable to the downturn in economic growth. Researchers fear that the fall in spending may speed up if industrial funding, which has remained stable, also begins to drop.

The proposed budget for 1994 does little to dispel such pessimism. The Ministry of Finance has imposed a cut of 1 per cent next year on all public spending classified as 'subsidies'. This cut, which will probably increase to 5 per cent in 1998, will affect the Dutch fundamental (NWO) and applied (TNO) research organizations and the Royal Netherlands Academy of Arts and Sciences (KNAW), but not the universities.

The way in which research funding became classified, improbably, as subsidies "is something that nobody understands", says Peter Tindemans, the director-general of the Ministry for Education and Science. The NWO and the KNAW have publicly protested against the decision. They have also asked Marius Cohen, who became secretary of state for education and science in July, and who is responsible for science policy, to change this classification. Tindemans agrees that it is inappropriate, but says that the ministry of finance is

reluctant to review the decision.

Research budgets are already under pressure because the government has abolished index linking. Moreover, it has transferred responsibility for paying employment benefits from the Ministry of the Interior to the research organizations and universities. The government has accompanied the measure with extra money, but many feel that this is less than the true additional costs.

Hein Meijers, a spokesman for NWO, says that the measure will also discourage research organizations and universities from hiring postgraduates and postdoctoral fellows because they would have to pay them benefits if they did not find jobs afterwards.

University budgets would suffer further if, as proposed, they take over responsibility for maintaining their buildings from the government. But again, the budget proposed to accompany the measure is insufficient. Unless it is increased, universities would have to choose between letting their buildings deteriorate and diverting money from other budgets.

The Netherlands spent DFL10.9 billion on research last year. The new cuts will amount to around DFL5.5 million next year and DFL67.3 million in 1998. These may be offset, however, by the government's plans to spend around DFL250 million on research from a special infrastructure fund which has been set up using extra income from the country's natural gas resources.

The government has also announced that it will let companies pay 10 per cent less income tax on their contributions for staff doing research. Researchers have generally welcomed the move, although some say that companies might simply pocket the savings — an estimated DFL350 million a year.

Declan Butler

French debate cools over nuclear waste storage

Paris. Public opposition to the French government's plans for storing high-level nuclear waste appears to be evaporating. Four years ago the government abandoned plans to drill four candidate storage sites because local opposition spilled over into riots. Now as many as 30 local authorities are clamouring to host sites.

The main cause of this shift is a decision announced by the government on 30 December 1991 to impose a 15-year moratorium on deep-storage of long-lived nuclear waste; such waste will continue to be stored on site for the time being. The moratorium has bought the government time to discuss the issues with the public in a more open and relaxed atmosphere.

This contrasts with the situation during the 1980s, when the National Agency for the Management of Nuclear Waste (ANDRA), then part of the French Atomic Energy Commission (CEA), pursued a policy of deep-storage as the exclusive solution to managing nuclear waste.

Last year, the government split ANDRA off from the CEA, and appointed foreign scientists to its scientific board. Now Christian Bataille, a member of parliament whose report shaped the 1991 law, has begun negotiating with local authorities to select sites next year for two underground laboratories.

ANDRA proposed building these test laboratories over five years, and then to carry out geological tests for a further eight. A decision on whether to pursue deep-storage, and if so where, would not be taken until 2006. In addition, the government has promised to study ways of reducing the volume and longevity of waste in the interim period, for example by transmutation to shorter-lived elements (see *Nature* 365, 381; 1993).

Many feel that deep-storage remains the best solution, and are sceptical of the technical feasibility of alternatives. But they also agree that the commitment has reassured the public, and that the attention to new technologies may bring benefits.

The government has also said it will consider designing any storage sites — which would be built around 2016 — in such a way that the waste could be easily retrieved if future technologies allow it to be treated.

Concern over rising unemployment, as well as the prospect that underground laboratories will bring local jobs and investment, has been the other main reason for the enthusiasm of local authorities. Such arguments have been aided by the political isolation of the Greens following their unexpected poor showing in the general elections earlier this year. **Declan Butler**

Russia heads for CERN membership

Munich. Four months after declaring its interest in becoming a full member of the European Laboratory for Particle Physics (CERN), Russia has signed a scientific and technological cooperation agreement that will prepare the way for membership in the near future. It has also pledged its support for CERN's plans to construct the Large Hadron Collider (LHC).

The three-year agreement was signed in Moscow on 30 October by the Russian science minister, Boris Saltykov, and CERN's director-general, Carlo Rubbia. It is seen as a transition period between the long-standing collaborations of the past 30 years and full membership.

Full membership is at present unreal-

istic because Russia would not be able to afford the fees. Russia's particle physics programme has been almost paralysed by the collapse of the Russian economy since 1990. For example, plans to extend the power of Russia's largest accelerator, the UNK, housed at the Institute for High Energy Physics in Protvino near Moscow, have had to be scaled down from 3,000 GeV to 600 GeV, even though the tunnel has been built.

The umbrella agreement has no specific sum of money attached to it. But Saltykov has promised at least to maintain Russia's current level of commitment to particle physics research both at home and at CERN in Geneva. **Alison Abbott**

Shanghai takes steps to boost scientific skills

Beijing. The city of Shanghai has taken a series of independent initiatives aimed at securing its primacy as a centre for Chinese science and technology. But its actions have also raised fears of growing regional imbalance of research funding in China.

The city has just held a major, biennial science and technology fair, and has set up its own centre for human genome research. Shanghai has also announced the doubling of a loan scheme to support basic research in the city, a training scheme for young scientists and special bonuses for outstanding young researchers.

The two-week fair, which opened on 18 October, was held for the first time in 1991, and will now take place every two years. Its purpose was to bring home to every citizen of the city the importance of science and technology, and it featured exhibitions, academic symposia and various popular science activities. During the fair, all of the city's major higher and research institutions were open to the public, with scientists standing by to explain their work.

The proposed Shanghai Key Laboratory for Human Genome Research received approval from the Shanghai Commission of Science and Technology on 1 October. The creation of the laboratory may have important repercussions for the management of research in a country which is just beginning to break away from the usual strict central control over scientific institutions.

In particular, although the Shanghai facility was launched just after China had announced a national human genome project (see *Nature* 365, 200; 1993), it remains very much Shanghai's own initiative, and its research plan, funding and management will not be affected by the national initiative.

The extra money for researchers in the city will come from the expansion of an agreement signed in 1991 by the Shanghai Commission of Science and Technology and the Shanghai Industrial and Commercial Bank. The bank has established a loan fund that has channelled nearly 40 million Yuan for 1991–93 to support research. On 15 October, the two sides agreed to provide an additional 60 million Yuan to the fund in the next two years (1993–95).

Science administrators will try to use the money to strengthen areas of research where Shanghai is already strong. Biological, materials and information sciences are three areas earmarked for additional funding; a total of 14 projects in those fields have been selected for priority support, each receiving 300,000 Yuan.

The commission has also launched a three-tier young scientist training scheme, aimed at 'general' young scientists, 'key'

young scientists and 'principal' young scientists, who will get different levels of financial support.

With backing from the municipal bureaus of finance and personnel, the city will select a hundred young scientists each year



Already a busy harbour — busy laboratories too?

to receive a special monthly bonus ranging from 300 to 400 Yuan — higher than the monthly salary of most Chinese scientists.

Those who have worked with projects

funded by the National Science Foundation of China and by the Shanghai Science Foundation are eligible for consideration. The aim is to counteract the attraction of commercial jobs, which are thought to be taking some of the best basic researchers away from science.

And Shanghai has painstakingly attempted to create a favourable infrastructure for research in the city. Its Pudong District has given priority to high technology, and has become a key growth area for Shanghai's industry. The new development zone is the home of a national Chinese high technology corporation, a conglomerate involving the country's 609 principal universities. The city's technology exports now go to more than 30 foreign countries, making an annual profit of \$250 million.

You Qin Li

Genetic testing under scrutiny in US

Washington. Existing policy guidelines governing the use of genetic testing need an urgent overhaul in order to stress the importance of voluntary participation, informed consent and the need to protect confidentiality, according to a report released last week by a committee of the Institute of Medicine of the National Academy.

In its two-volume report, *Assessing Genetic Risks: Implications for Health and Social Policy*, the committee identified a number of pressing concerns and outlined its recommendations, including the need for greater federal supervision of genetic testing laboratories, and improved quality control of genetic tests.

The committee also called for safeguards to protect individuals against discrimination from health insurers and employers, and advocated more rigorous education and training of health care providers in the area of genetic testing and counselling.

The majority of committee members agreed that there was a need for a broadly representative and independent national advisory committee that would report to the Secretary of the Department of Health and Human Services and to Congress.

The basic message of the report, according to Neil A. Holtzman, professor of paediatrics, health policy and management and epidemiology at Johns Hopkins University Hospital, is that "the committee places patient autonomy above all else".

According to the committee, prenatal testing raises some of the most difficult issues: the ability of physicians to diagnose genetic disorders of the fetus far exceeds any ability to cure them; a woman's reproductive choices are more limited at this

stage, and the occurrence of false-positive results can have serious implications.

For these reasons, the committee felt that women undergoing prenatal testing should be fully informed about the benefits and risks of the testing procedure, the possible outcomes, and the available options.

All counselling, which should be given before and after testing, should be of a "non-directive" nature; decisions about whether or not to continue with a pregnancy should be left to the individual.

The committee strongly opposes the use of prenatal testing for the pursuit of eugenic goals, such as determining the sex or other non-disease oriented traits of the fetus. And although the committee recommended that all screening — including that of newborns — should be voluntary it did feel that, following the careful evaluation of pilot studies, state health departments could mandate the "offering" of tests for diagnosing treatable conditions in newborns.

Where newborns being tested for disorders would clearly benefit from early diagnosis and treatment (for example, in the metabolic disorders phenylketonuria and congenital hypothyroidism), some committee members felt that a case could be made for mandating genetic testing.

The committee urged caution over the use of genetic tests to determine a person's susceptibility or predisposition to genetic disorders that can occur later in adult life. For single-gene disorders, the committee said that population screening should be restricted to conditions of a relatively high frequency and that are either be preventable or treatable.

Diane Gershon

Recipe for innovation

SIR — Your leading article on research foresight (*Nature* 365, 679–80; 1993) pictures the possible ruin of British research by doltish industrialists, as they try to recover from their past neglect of the opportunities offered them by basic research. There is a lot wrong with UK industry, but useful criticism needs a better understanding of the process of innovation than the one underlying your picture.

Innovation doesn't happen by industry recognizing independently a bright idea from basic research, backing it with money and bringing it to market. As shown by the examples of electronic devices based on semiconductor research and of pharmaceuticals based on molecular biological research, innovation actually results from a close interaction between basic research and industry. Innovation requires that basic researchers learn something about development, production, marketing and finance, without weakening their curiosity-driven research skills — hard work, but rewarding, intellectually and sometimes financially.

The chief purpose of research foresight activity ought to be to promote such contact between researchers and people from industry, aimed both at identifying exciting science and the means for its application. If it is to work well, interaction should be between people active at the bench and in the market, more than between senior academic administrators and industrial directors. As you say in your article, it will have to be a democratic process, in which people participate because it helps their work, rather than because it is felt to be politically expedient.

Basic researchers should therefore support the process of research foresight but try to make it a genuine process of interactive learning. If they succeed, so will UK industry, and then the case for better public funding of basic research will become irresistible.

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Slide viewer

SIR — In January 1992, the habit of molecular biologists to present gels as sole results of their research led John Maddox to ask: "Is molecular biology yet a science?" Twenty months later, when attending a conference on molecular chaperones, I realized that his call for more quantitative analysis of the living cell (*Nature* 355, 201; 1992) has passed

unheard. Speakers with backgrounds in cell or molecular biology still present incredibly long series of gels, which, apart from being ugly and streaky, can neither prove nor explain anything.

Some self-reflection on the question "why do we scientists present slides?" leads us to a distinction between two main kinds of pictures: those presenting evidence, and those explaining concepts too complicated to be easily grasped from oral presentation. While gels have a strong negative efficiency with respect to the second task — they don't provide explanations but they make more (visual) explanation necessary — they achieve poor ratings in the first one. The presence of a band for compound X and the absence of Y in a gel do not prove anything to the disbeliever — for instance, if the ratio of concentrations is one to three, one can easily stain a gel so that the absence of the minor component is suggested. A densitometric profile of the same gel would at least give a quantitative estimate about what is to be understood by 'absent' and 'present' (one could claim, for example, X to be at least in hundredfold excess over Y). And, if this result is to be appreciated by anybody who has not studied X and Y for more than 10 years, this picture should be preceded by a didactic cartoon explaining why we do (or do not) expect to find X present and Y absent, and what the implications would be, if we found a different result.

In summary, photographs of simple stupid SDS-gels should be banned from conferences and journals. Pictures are a crucial means of communication among *Homo sapiens*, so we should waste at least as many thoughts on them as on our words. Of course, there are more goals to the "next slide please" than the two discussed, and one of the others is to make people laugh. This, at least, was inadvertently achieved by one of the contributors at the conference mentioned above with a gel: he presented a single lane of a gel with a single black band on it. Economically thinking, he could use this slide in every talk to "prove" anything.

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Understanding of science

SIR — Carmen Pryce is unfair to the journal *Public Understanding of Science* in her review (*Nature* 365, 589; 1993). The journal does not claim to be a magazine for the public, but an academic journal in which those undertaking research in issues in public understanding of science can communicate and share their thinking and

results. That process is an essential part of scholarship, and the journal should be judged in that light.

Pryce asks if such a journal is necessary. The Committee on the Public Understanding of Science (COPUS), a body seriously concerned with developing and initiating new activities that promote a greater understanding of science and its methods, can assure her that it is. For us it is important that our schemes are solidly based on an understanding of how the 'public' receives and interprets its information. Educational research has shown the inadequacy of a simple teacher-pupil model. We are also required to evaluate the way in which our activities achieve their ends, an aspect where similarly little research has been done.

Interpreting research findings in public understanding of science is not always easy for those outside the academic discipline, but public understanding is hardly alone in that. Nor does it detract from the importance of the research or the need for a journal in which such results can be communicated.

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Compulsory read?

SIR — *Nature* is tempted to involve "all of Darwin" to defend evolution from California creationists (*Nature* 364, 746; 1993). To begin with, I suggest that Darwin's *The Origin of Species* and *Journal of Researches* be made compulsory reading for the students of California. The risk, of course, is that the former may put many students to sleep while the latter, which was Darwin's favourite, is much more lively and better written. Moreover, it contains inspirational passages about Christian principles and an essay about the hollow conical pitfalls of the lion-ants of England and Australia, which reads, in part:

There can be no doubt that this predacious larva belongs to the same genus with the European kind, though to a different species. Now what would the sceptic say to this? Would any two workmen ever have hit upon so beautiful, so simple, and yet so artificial a contrivance? It cannot be thought so: one Hand has surely worked throughout the universe. (*Voyage of the Beagle*, 325; Penguin 1989).

Yes, let "all of Darwin" be used to defend evolution from California creationists.

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Did Popper solve Hume's problem?

Allan Goddard Lindh

How do we know the Sun will rise tomorrow? Hume's concern raises two questions: the problem of empirical inference was solved in this century by Popper; an approach to the stability of nature in time is outlined here.

The sun will not transgress his measures; otherwise the Furies, ministers of Justice, will find him out.

Heracleitus of Ephesus

In his discussions of David Hume's problem of induction¹, Karl Popper writes as if it were only a single logical problem: "the logical problem of induction arises from Hume's discovery . . . that it is impossible to justify a law by observation or experiment, since it 'transcends experience'². And yet induction clearly involves at least two distinct logical problems: Hume was also concerned with the possible instability of nature in time, a concern with implications that extend far beyond induction, threatening critical rationalism at its core. Clearly, the first assumption involved in extrapolating experience into the future is that the nature of reality will not change; today, we would formulate the problem as whether the laws of physics, and the relevant physical constants, will remain stable in the future. In Hume's words (my italics):

If there be any suspicion that *the course of nature may change*, and that the past may be no rule for the future, all experience becomes useless, and can give rise to no inference or conclusion. It is impossible, therefore, that any arguments from experience can prove this resemblance of the past to the future; since all these arguments are founded on the supposition of that resemblance³.

and

. . . and that *the course of nature continues always uniformly the same* . . . We can at least conceive a *change in the course of nature*; which sufficiently proves, that such a change is not absolutely impossible⁴.

It is clear from these and other passages that one of Hume's primary concerns was the stability in time of the physical world; I will refer to this as 'Hume's problem'.

Popper, however, is concerned with a very different problem of induction: the question of whether our observational results are sufficient to extrapolate our experience to other circumstances. He introduces this problem with a quotation from Max Born:

. . . no observation or experiment, however extended, can give more than a finite number of repetitions . . . the statement of a law — B depends on A — always transcends experience. Yet this kind of statement is made everywhere and all the time, and sometimes from scanty material⁵.

Born, however, makes no mention of any change in the laws of nature over time — "that the course of nature may change": he is clearly referring to the fact that no matter how many trials have been made of

an experiment, they are never sufficient to guarantee the result of the next trial. (Even a coin-tossing experiment that yields 1,000 heads in a row does not prove that the coin is two-headed, nor does it ensure that the next toss will result in a head; it only makes obtaining a head on the next toss highly probable.) I will refer to this concern as 'Born's problem'.

In his presentation of the problem of induction, Popper appears to be referring only to Born's problem — the empirical difficulty of extrapolating from a finite number of trials:

We can see this the moment we realize that the acceptance by science of a law or of a theory is *tentative only*, which is to say that all laws and theories are conjectures, or tentative *hypotheses* . . . So long as a theory stands up to the severest tests we can design, it is accepted; if it does not, it is rejected. But it is never inferred, in any sense, from the empirical evidence. There is neither a psychological nor a logical induction. *Only the falsity of the theory can be inferred from empirical evidence, and this inference is a purely deductive one*.

Hume showed that it is not possible to infer a theory from observation statements; but this does not affect the possibility of refuting a theory by observation statements. The full appreciation of this possibility makes the relation between theories and observations perfectly clear . . . and with it Hume's problem of induction⁶.

Popper's solution to Born's problem is very simple: given the logical asymmetry between verification and falsification, we can never know for certain whether any theory, law or generalization drawn from experience is true, but only that it has survived the best tests we can muster to date: we can only make suppositions about the results of additional trials. But there is no reference here to time, even though the concern "that the course of nature may change" is central to Hume's statement of the problem. Elsewhere, Popper is explicit on this point:

Although [Hume] often mentions an inference from the past to the future, we should, I suggest, take this only as a special case of the inference from what is actually known from observation to what is not known⁶.

and elsewhere

It is of comparatively minor significance whether such an inference from the observed to the unobserved is, from the point of view of time, predictive or retrospective; whether we infer that the sun will rise tomorrow or that it did rise 100,000 years ago⁷.

With all due respect, this suggestion does not seem sensible to me: Hume says in plain English that he is concerned "that the course of nature may change", that it is time he is concerned with. The solution

that Popper provides is independent of time and applies just as well to predicting the results of experiments performed in the past. So even if we accept (and I do) Popper's solution to the empirical problem of induction, his solution does not appear to be a solution to Hume's problem — the stability of the laws of nature in the future. Worse still, Hume's concern that the course of nature may change undermines Popper's falsification regime as well, for if the laws of physics change over time, a theory might appear false today and yet pass the same test tomorrow. Hume's problem remains, and it does not undermine just induction; it also undermines falsification, Popper's solution to Born's problem, the scientific method and all of critical rationalism.

Addressing Hume's concern requires two logically distinct questions to be answered, one that is subject to direct empirical testing, and one that is not: (1) are observations made in the past consistent with — do they even corroborate — the laws (and fundamental constants) of physics, chemistry, astronomy and so on that do not change with time? and (2) will comparable observations give the same results in the future?

When we consider when Hume lived, it is not hard to see why he might have emphasized the possibility that the course of nature may change; his *Treatise of Human Nature* was written in 1737, just 50 years after the publication of Newton's *Principia Mathematica*. Hume's evidence for the stability of the world in time would have included his personal experience of the seasons, gravity, the regularity of motion of the Sun, the Moon, stars and planets across the sky, the success of astronomers in predicting the times of eclipses and Halley's successful prediction of the return of his comet in 1682. But it seems extremely unlikely that Hume could have realized that we would someday be able to compute back from the current positions of the Sun, Moon and Earth to historical accounts of eclipses, as a test of the stability in time of the laws of gravity and of G , Newton's gravitational constant. This has now been done in a test that spans three millennia, placing an upper bound on the annual rate of change of G to about 1 part in 10 billion⁸. Similarly, the ratio of hydrogen to helium in the Universe has been used to estimate that the value of G was within 20 per cent of its current value during the first second after the Big Bang⁹, placing a comparable

limit on the average rate of change of G . Other indirect tests have been performed, to a lesser precision but spanning periods as long as billions of years^{10–16}; and such events as the observation of the expected neutrino burst from supernova 1987A, which occurred 186,000 years ago, provide checks on the stability of a wide range of physical laws and constants¹⁷.

Today we are not confined just to historical tests of G but also have laboratory and field tests of various physical laws and constants, some to an extraordinary precision over periods of years to decades¹⁸. So the first half of Hume's problem — the question of the stability in the past of the physical laws and constants — has been transformed into an empirical question, and while many of the critical experiments were not performed with this problem in mind, they have implicitly tested the stability of nature in time, and so far 'time' has withstood all challenges.

But given that we have today very precise empirical observations of the stability of the physical laws in the past, what does this say about the second half of Hume's problem, their stability in the future? The short answer is, I believe, that the empirical stability of physical laws in the past supports — albeit tentatively — the same stability in the future and thereby provides a (tentative) solution to Hume's problem. I will spend the rest of my time defending the proposition that there are now rational grounds for believing that the future will resemble the past.

First consider Occam's razor: "What can be done with fewer assumptions is done in vain with more"¹⁹. Fewer parameters are required to describe a universe in which the laws of nature are stable in time, than one in which the laws of nature vary; in the absence of any falsifying evidence, the simplest hypothesis prevails^{20–23} and nature is presumed to be stable in time.

But apart from an appeal to simplicity, do the laws of modern physics permit a stronger statement about the relation of past and future? In Feynman's words:

Space and time must be completely interlocked; after this discovery Minkowski said that 'Space of itself and time of itself shall sink into mere shadows, and only a kink of union between them shall survive'. . . in all the laws of physics that we have found so far there does not seem to be any distinction between the past and future. The moving picture should work the same going both ways, and the physicist who looks at it should not laugh²⁴.

All modern formulations of the laws of physics treat space and time as an isotropic homogeneous four-dimensional space within which there are no preferred directions. The invocation above of Occam's razor is equivalent to asserting that there is nothing special about this moment in space-time (translational invariance of the laws of physics in four-space), although this assumption already forms a corner-

stone of modern cosmology:

. . . while Einstein and other physicists were looking for ways of avoiding general relativity's prediction of a nonstatic universe, the Russian physicist and mathematician Alexander Friedmann instead set about explaining it. . . Friedmann made two very simple assumptions about the universe: that the universe looks identical in whichever direction we look, and that this would also be true if we were observing the universe from anywhere else. From these two ideas alone, Friedmann showed that we should not expect the universe to be static. In fact, in 1922, several years before Edwin Hubble's discovery, Friedmann predicted exactly what Hubble found²⁴.

Hubble's discovery provided a measure of corroboration for general relativity and, in a certain sense, for Friedmann's assumption of a homogeneous isotropic four-space, thus indirectly providing support for the stability of nature in time as well. Furthermore, translational invariance of the laws of physics in time also implies the conservation of energy²⁵; it is one thing to consider that the course of nature may change, but violating the first law of thermodynamics is quite another.

At the dawn of modern physics, Galileo's and Newton's assumption of the stability of nature in time was essential to their work, buttressed only by very limited observations. Since Hume's time, two lines of investigation have provided additional underpinning for their assumption: observations of the temporal stability of the laws of nature over a large sector of space-time and the intertwining of space and time in the work of Minkowski, Einstein, Friedmann and others. So although the invariance of the laws of nature to translation in space-time might still retain the character of an assumption, its place as the cornerstone of the entire edifice of modern physics makes it — in a certain sense, the best corroborated of all the laws of nature.

This situation represents an example of a metaphysical proposition, which was untestable in Hume's time, being transformed by advances in scientific understanding and technology into a scientific conjecture that has already survived an extraordinary series of tests. Whether the apparent stability of the laws of nature in the past actually implies that these laws will be stable in the future depends on the depth of one's belief in the essential nature of Occam's razor in the rational exploration of the Universe — a metaphysical question that remains an active area of discussion^{1,20–23,26–28}. Of course, for those who believe that Occam's razor forms a cornerstone of all rational inquiry, the question of the stability of nature in time is moot, for without Occam's razor there can be no science to assess the stability; this metaphysical proposition will be true, I believe, in any conceivable future. So we have in essence reduced the metaphysical underpinnings of modern science by one assumption: Occam's razor plus the

assumption of stability in time has been replaced by Occam's razor plus observations of stability in the past.

In a testimonial²⁹ to Popper on his ninetieth birthday, Hermann Bondi quoted Popper as saying: "We advance like a person walking through a swamp, first painfully pulling up one leg and advancing it, and then the other. One leg is labelled 'technology' and the other 'science'". What he neglects to add is that on our journey through the swamp we drag a bundle labelled 'metaphysics', which is transformed by the experience. Having said this, it is only fair to give Popper the final word and to thank him for his long, extraordinary service to humanity in keeping alive the torch of Socrates:

I believe that it would be worth trying to learn something about the world even if in trying to do so we should merely learn that we do not know much. This state of learned ignorance might be a help in many of our troubles. It might be well for all of us to remember that, while differing widely in the various little bits we know, in our infinite ignorance we are all equal²³.

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Has nature overwhelmed nurture?

There is a danger that too zealous and uncritical advocacy of the importance of human genome studies to the exclusion of others may provoke disbelief, distrust and resentment.

THERE is a possibility that the most damaging pitfall in the path of the new genetics, and especially the Human Genome Project in its various manifestations, is none of the ethical questions so far raised, but the apparently inescapable triumphalism that accompanies major bursts of discovery. As new genes are found and linked with various of the human conditions by the study of family pedigrees in which those conditions are conspicuous, the general press is littered with news of the discovery of the gene 'for' this or that, haemophilia or even excessive aggression in males. One unexpected and unfortunate consequence is a growing belief in a kind of genetic predestination.

It is now literally 40 years since it became plain that inherited genes are not only the raw material for an organism's genetic endowment of its descendants, but that they also embody the biochemical programs by which cells of an organism conduct their business. That lesson seems generally to have sunk in, which is a triumph of a kind for the popularizers of science. The danger is that it may have sunk in too well, leaving people in general with the impression that not merely their physical well-being but also their behaviour is determined by the genes they happen to have inherited.

The implications of that proposition for the general conduct of human affairs are clearly profound. The possibility of predicting, with the help of appropriate genetic markers, that an individual will or will not contract one of several inheritable diseases on a quickly growing list thereof has already provoked fierce arguments (about health insurance and discriminatory employment, for example) which are not intrinsically novel, but which are likely to be increasingly clamant in the years ahead.

The Strong Genetic Principle, that every aspect of the human condition is predetermined by the genes, must inevitably raise more sound and fury. Will the time come when an abnormal nucleotide sequence is a sufficient defence in the courts against, say, a murder charge? When we know the gene 'for' kleptomania, will similar pleadings also set burglars free? Will what used to be moral philosophy and much of what is now called sociology be subsumed in the human genome projects? And how long will it be before the clever people in charge of those enterprises are able to predict, from a close examination of their sequences, the future of the novel as an art form?

These questions, put starkly in that form, will seem to many serious people to be so

absurd as to deny the Strong Genetic Principle. But the confidence exuded by the genome projects leaves many others in doubt. And many who should know better have, sometimes in unguarded comments on their own discoveries, given comfort to genetic predestinarianism. But geneticists in particular, with the arguments about nature and nurture of the 1930s (on Hitler's Aryan concept) and 1960s (about the inheritance of IQ) fresh in their minds, should be more vividly aware than most that the Strong Genetic Principle is a fallacy.

Moreover, there is ample evidence, of a kind that will be as easily appreciated by the world at large as the doctrine of inherited DNA as a repository of inherited biochemical programs has been, to show that nurture still has a place in moulding the nature of an organism. There are many familiar proofs in the textbooks that a cell's DNA does not unambiguously determine its character.

On present understanding, for example, liver cells and lymphocytes from the same individual have identical complements of DNA, but very different properties. The present explanation is that different sets of genes are permanently activated in the lineages yielding the two different kinds of cells. The mathematicians would say that the relationship between DNA and phenotype is not necessarily single-valued. In the nematode and even *Drosophila*, the process of differentiation seems neatly hard-wired, although the molecular mechanisms that spell the differences between lineages have not yet been identified.

As it happens, the influences on undifferentiated cells that determine which lineage they follow are, in the strict sense, environmental, the secreted products of homeobox genes among them. Who at this stage can be sure that in more complicated (or less well studied) organisms, the same mechanisms may not allow for the moulding of the idiosyncrasies of an individual organism, perhaps even under environmental influences external to itself? In other words, there may ultimately be ample and even definable room for nurture.

The vivid textbook illustrations are the many accounts of how even organisms consisting of single cells can change their external form dramatically under different conditions. Some bacteria form viable spores when, for example, dehydrated, but their complement of DNA is not changed. Some yeast species even change their reproductive methods under environmental influences. That *in utero* influences can pro-

foundly affect newborn babies (as in spina bifida, for example) is well documented. Is it likely that some of the most labile aspects of the human phenotype, those constituting personality, will be exempt from similar and more subtle influences?

What has been learned in recent decades about the plasticity of the newborn mammalian brain points directly to one point at which nurture must exert leverage. At least in the shaping of visual function, if the brain is a computer of a kind, its interconnections must be determined by experience of the uses to which this faculty will be put. Is not the same likely also to be true of other faculties, notably those constituting people's personality, whatever that may mean? And who will be surprised if the duration of plasticity, during which crucial interconnections are established, lasts longer than the few months characteristic of the visual system?

Classical genetics already provides latitude of this kind in the concept of the 'penetrance' of a gene. A recessively inherited condition such as sickle-cell anaemia can be unambiguously related to the underlying nucleotide sequences; two copies of the sickling globin gene generate red blood cells with the sickling habit and, as a consequence, the symptoms of anaemia. But the link between genotype and phenotype is not always unambiguous. A genotype may be a necessary, but not a sufficient, condition for the phenotype; the individual concerned inherits only a *susceptibility* for the phenotype. Penetrance is then simply the statistical chance that an individual inheriting the genotype in question will exhibit the associated phenotype. Of course, the strong genetic principle would be recovered if there were found to be other genes than those first identified that turn susceptibility into certainty, but (at this stage) it would be rash to deny that the missing ingredients may be aspects of nurture.

Especially in relation to human behaviour, it is possible to exclude the influence of nurture only by dismissing as unimportant a century of psychology and psychoanalysis. Although much of that may leave a lot to be desired by modern standards, it seems improbable that it can be entirely content-free. Most geneticists will probably agree that nurture still has a place. But to fail to say as much, explicitly, may generate more disbelief and even resentment than ethical worries have yet engendered. That would be a misfortune for a programme of such high promise.

John Maddox

Down under the volcano

Jonathan Fink

In the past two years, more geologists have been killed studying active volcanoes than ever before. It was perhaps fitting then that a recent meeting* of over 600 volcanologists was held in the relative calm of Australia, a continent that has seen no eruptions in historic times. Under the theme of "Ancient Volcanism and Modern Analogues", speakers discussed novel ways to learn about the normally inaccessible interiors of incendiary pyroclastic flows and giant submarine landslides, in order to improve prediction of their behaviour and reduce the associated loss of life.

In Japan, the collapse of Mount Unzen's lava dome on 3 June 1991 sent a cascade of intensely hot magma fragments down the flank of the volcano. Although the most extreme conditions occurred within the dense, concentrated pyroclastic flow, most of the victims were killed or injured by the cooler, more dilute pyroclastic surge cloud. Harrowing accounts provided by the handful of survivors formed the basis for an unusual study (S. Aramaki, Hokkaido University). From observations such as "Mr A heard a noise in his yard, opened the front door, and was immediately knocked on to his rear end", and "Racing away from the eruption, Mr C saw red glowing pumice lumps bouncing on the hood of his car", it was possible to map out the overpressures and temperatures within the ash cloud. The fact that nearly all who perished were outdoors and enveloped by the more extensive surges rather than the hotter but more restricted pyroclastic flows prompted a recommendation that numerous concrete shelters be built around the base of the volcano as shields against these more widespread dangers.

Recordings

If Mr A and Mr C had intended to be in the path of a pyroclastic cloud, they could have been outfitted with asbestos suits, thermocouples and pressure gauges to quantify conditions hidden within the infernal avalanche. A safer approach (M. Sumita, University of Kiel, and J. Oikawa, University of Tokyo) relies on the latest acoustic technology to record precisely the sounds made by pyroclastic flows. Comparing frequency spectra of the whooshes, clinks and crashes made by colliding pumice fragments with data from experiments in which steel balls of different sizes were poured down a slope, Sumita calculated that average particle diameters decreased by 30 per cent during

30 seconds of travel. This kind of information provides new constraints on how pyroclastic flows are generated and advance.

Although the Unzen eruption has continued for nearly three years, most residents of nearby Shimabara have been only slightly inconvenienced. Not so the Mayans living 1,400 years ago in Ceren, in what is now El Salvador. New archaeological and volcanological findings (C. D. Miller, U.S. Geological Survey), show that a rapid series of explosive eruptions took place within 2 kilometres of the village when rising magma encountered ground water. Buildings were burned, blasted down and then buried by up to 5 metres of ash and pumice which preserved details of Mayan life rivalling the Roman ruins at Pompeii. Dirty dishes, pots full of food, the remains of a duck still tethered to a stake, and footprints in ash all suggest a precipitous evacuation of the town, yet so far no corpses have been found. It is not known if the citizens of Ceren understood volcanic unrest well enough to respond to its precursory signs, or whether future excavations will show their remains lying in some as yet undiscovered tomb.

Pyroclastic flows like those at Unzen and Ceren are driven in large part by gravity. Work at two of the world's most active volcanoes, Kilauea and Etna, has shown that gravitational effects can sometimes be far more catastrophic (from the standpoint of the volcano) than those associated with such pyroclastic flows. Studies using the Global Positioning System satellites have shown that entire mountains can be torn apart when magmatic injection causes flanks to slide off towards the ocean; the giant landslides can involve huge amounts of material covering thousands of square kilometres, and can cause very large tsunamis and earthquakes.

Before now, the factors controlling the speed of this slumping have been elusive. New petrological examination of samples from the submarine extension of Kilauea's East Rift Zone (D. Clague and R. Denlinger, U.S. Geological Survey) indicates that the giant landslide is slipping on an unusually concentrated slurry of olivine crystals that precipitated out of the basaltic magma to make a basal lubricating zone "as fluid as duck droppings". This surprising result suggests that the sides of the most active volcanoes can spread like glacial ice advancing into the sea.

Similar collapse has also been recognized at Mount Etna, where the Valle del Bove represents the scarp of a massive landslide that extends eastwards into the Mediterranean. Detailed structural and

geophysical mapping (W. McGuire and colleagues, University College London) has revealed a series of small, seaward-sloping faults that act as brakes on the larger-scale movement. When these faults are free to slip, the volcano sags, allowing more voluminous eruptions from the summit and rift zones. When friction causes the faults to lock, eruptions are suppressed and intrusive injection extends to more distal parts of the volcanic edifice. Thus settling of crystals and movement along seemingly minor faults may be competing influences on the efforts of gravity to reduce huge volcanoes to piles of submarine rubble.

Volcanic sediments

At some stage in the development of all volcanoes, gravity wins and the many types of rock that make up the construct are broken apart and transformed into sediments. Most volcanologists have assumed a simple correspondence between individual volcanoclastic layers and discrete eruptive events. But the conversion of a coherent volcano into an assemblage of loose sediments had never been fully documented until P. Kokelaar (University of Liverpool) took an interest, looking at White Island in New Zealand and Stromboli in Italy.

Combining data from submersibles, scuba dives, dredging and sonar, Kokelaar mapped out the exceedingly complex path by which lava, ash and pumice fragments first become comminuted as they roll downhill, are winnowed as they pass through the filtering effect of the shoreline, then become ponded in offshore stores where reworking by storms is common, and finally become consolidated in deeper deposits. Demonstrating how this perspective can radically alter interpretations of submarine volcanic sequences, he showed how a previously mapped debris fan off the coast of Stromboli is three times larger than the concavity that was its apparent source, and concluded that the deposits were formed by three discrete events.

After the latest research conclusions are reported, volcanological meetings inevitably turn to consider ways to reduce the societal impact of future eruptions. This year such concerns were overshadowed by questions about how the safety of volcanologists themselves can be better guaranteed. Although participants admonished each other to think twice about taking nonspecialists into potentially active craters, there was general recognition that substantial risks will remain for volcanologists in the field as long as we know so little about the factors controlling so wide a range of hazardous volcanic processes. □

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Structure and selectivity

Gary Yellen

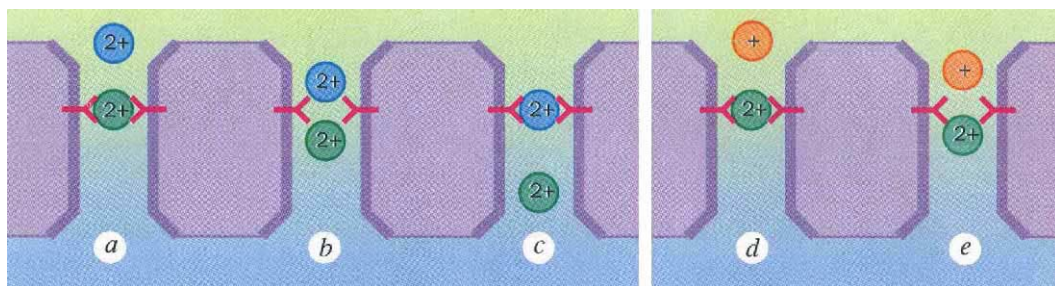
SELECTIVE permeation of calcium ions across cell membranes is central to the action of calcium as a second messenger, yet the mechanism by which voltage-dependent calcium channels produce this selectivity has been a puzzle. The latest refinement in the concept of calcium selectivity is found in reports by Yang *et al.* (page 158 of this issue¹) and Kim and colleagues², which take us closer to an appreciation of the structural basis of the phenomenon.

The conceptual problem with calcium selectivity is this. All ion channels must

concentrations, the Ca^{2+} itself begins to carry current.

These findings led to an ingenious proposal^{3,4} to explain calcium channel selectivity, based on the idea of obligatory single-filing of ions through channels. The 'two-site' model posits the existence of two high-affinity, ion-binding sites in the channel, each of which can bind either Na^+ or Ca^{2+} . In the absence of Ca^{2+} , Na^+ conducts current through the channel. When a small amount of Ca^{2+} is added, it prevents Na^+ current by binding with even higher affinity to one of the sites.

In multi-ion calcium selectivity, the negatively charged site (red) in the channel can contain either one Ca^{2+} ion with high affinity (a) or two ions with lower affinity (b). Even at low $[\text{Ca}^{2+}]$, the site is occupied by one Ca^{2+} ion (green). For net Ca^{2+} movement to occur (a–c), a second ion approaches (a), shares the site with the original ion (b) and, by reducing its affinity, permits it to leave (c). In the presence of Ca^{2+} , monovalent ion (orange) permeation cannot occur (d, e). A monovalent ion can share the site with the Ca^{2+} ion (e) but because it binds more weakly it always unbinds before the Ca^{2+} ion, in the direction from which it came (d).



perform a delicate energetic balancing act to allow ion permeation. Through favourable binding interactions with the ion, they must compensate for the extremely unfavourable energy of moving a charged ion through the low dielectric of the lipid membrane. But they must not overcompensate: if the binding is too tight, then the throughput of the channel will fall below the 10^6 – 10^8 ions per second that is characteristic of most ion channels. Intuitively, it seems that a cation channel should have some site with at least partial negative charge; yet it is hard to design a charged site that would bind the highly charged Ca^{2+} ion rapidly and reversibly without also allowing permeation of the less-charged monovalent ions Na^+ and K^+ . Because one supposes that Ca^{2+} would bind better than Na^+ to a negatively charged site, it should permeate even more slowly.

The first step in understanding calcium channel permeation came with the discovery that calcium channels have, in fact, not solved this problem^{3,4}. In some ways they are very unselective: in pure solutions of NaCl or CaCl_2 , Na^+ ions actually conduct better than Ca^{2+} ions through the calcium channels. Only when Na^+ and Ca^{2+} ions are mixed do the channels appear quite selective for Ca^{2+} . The calcium selectivity is phased in as $[\text{Ca}^{2+}]$ is increased in the presence of physiological $[\text{Na}^+]$: at micromolar concentrations of Ca^{2+} , the sodium current is blocked; at millimolar

Na^+ can still bind to the second site, but it cannot displace the tightly bound Ca^{2+} (and it can't get around it, either). Finally, when levels of Ca^{2+} are high enough, a second Ca^{2+} ion will occupy the channel. It is assumed that in the doubly Ca^{2+} -occupied state, the binding affinity of both Ca^{2+} ions is reduced by electrostatic repulsion, so that one of them will leave the channel quickly. By alternating between single and double occupancy by Ca^{2+} , the channel can permit rapid Ca^{2+} movement and yet exclude the weaker-binding Na^+ ions. The two-site model — originally considered in detail in the 1970s (ref. 5) — also predicts other peculiarities in Ca^{2+} channel permeation and blockade^{6,7}.

An even simpler scheme, with only a single site (but still with two ions in single file), was proposed last year⁸. This single site can contain either a single Ca^{2+} ion with high affinity, or two with low affinity (see figure). Formally, this model behaves in the same way as the two-site model, but it suggests a different physical picture: rather than two distinct binding sites placed at just the right distance for interaction, it envisages a single, highly attractive site capable of holding two ions in close proximity, but located in a narrow, single-filing region of the pore. Support for a version of the single-site model comes from beautifully detailed studies⁷ on ion interactions in the pore; these data support a picture of a closely spaced and

sometimes degenerate 'set' of high-affinity sites near the extracellular surface of the channel, probably consisting of several carboxylate residues.

Insight into the structural underpinnings of the Ca^{2+} ion-binding sites came first from site-directed mutagenesis experiments on the closely related voltage-dependent Na^+ channel. The voltage-activated K^+ , Na^+ and Ca^{2+} channels are members of a homologous superfamily; the K^+ channels are tetrameric, whereas the main subunit of the Na^+ and Ca^{2+} channels contains a fourfold repeat of the K^+ channel motif. Alignment among various Na^+ and Ca^{2+} channel domains, in a region previously found to contain a site capable of affecting blockade by tetrodotoxin, reveals a set of four homologous-

ly positioned glutamate residues (one in each domain) in the calcium channel. The homologous four positions in the Na^+ channel contain aspartate, glutamate, lysine and alanine. Replacement of the last two residues with glutamate had dramatic results⁹: the modified Na^+ channels are blocked by micromolar Ca^{2+} and can conduct some current at 10–100 mM Ca^{2+} . Selectivity among monovalents is practically eliminated by the mutations. The implications for the normal selectivity of Na^+ channels are still unclear. But the results strongly indicated that the structural basis of Ca^{2+} channel selectivity resides mainly in this ring of four negatively charged glutamate residues.

This prediction is borne out by the new work^{1,2}. Mutation of the glutamates alters the monovalent/divalent selectivity of the channels, as well as the affinity of blockade by divalent ions (including Ca^{2+} blockade of Li^+ current). If there are two distinct sites with similarly high affinity, they must both be influenced by the glutamates, because the high-affinity block can be nearly eliminated by their mutation.

Another nuance noted by both groups is that the mutation of the individual glutamates does not have exactly equivalent effects. This result is not entirely unexpected — the four domains of the calcium channel are homologous but very different in sequence, even in the region around the glutamates. But Yang and colleagues¹

propose the interesting hypothesis that the broken symmetry of the glutamates may be functionally important for Ca^{2+} permeation. In other words, a perfectly symmetrical ring of glutamates might not have the flexibility required to accommodate either one or two Ca^{2+} ions. By misaligning the four glutamates, the site may become more spread out in space and more fuzzy in its logic, now able to contain one Ca^{2+} with high affinity, or two with lower affinity. Further tests of the mutant channels, using the approach of Kuo and Hess⁷ to dissect ion-ion interactions in the channel, should help to reveal how qualitatively distinct are the roles of the individual glutamates.

Has nature already tried making Ca^{2+} channels from symmetrical channels and failed? The cyclic nucleotide-gated (CNG) channels found in photoreceptors and olfactory neurons are nonselective cation channels that are blocked by Ca^{2+} with moderately high affinity. Homotetrameric voltage-activated K^+ channels can, remarkably, be converted to match this description by deletion of two amino acids adjacent to an aspartate residue in a position homologous to the Ca^{2+} channel glutamates¹⁰. Although these channels have some of the salient features of Ca^{2+} channel permeation, they lack the most obvious: large Ca^{2+} currents. The CNG channels of the rod (which are not necessarily homotetramers) do carry about 15 per cent of their current as Ca^{2+} and the rest as Na^+ (ref. 11), but this mixed permeation is incompatible with the accepted calcium channel mechanism: when enough Ca^{2+} is present to carry substantial current, Na^+ current should be completely suppressed. It will be interesting to see if symmetrical Ca^{2+} -selective channels can be made by engineering of the CNG channels or the mutant K^+ channels, or whether — as Yang *et al.* suggest — a good calcium channel needs a slightly fuzzy binding site. □

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New light on stem growth

Winslow R. Briggs

BLUE light profoundly affects growth and development of higher plants, being responsible for a host of reactions including phototropism, inhibition of stem growth, promotion of leaf expansion and induction of gene expression. The longer-wavelength plant photoreceptor phytochrome has been identified but the blue-light photoreceptor has proved more elusive. A major step forward is now reported on page 162 of this issue¹, where Ahmad and Cashmore offer molecular and genetic evidence that a protein with strong structural homology to DNA lyase may be a photoreceptor involved in inhibiting stem growth.

The beginnings of modern research on phototropism were ushered in over a century ago by Charles and Francis Darwin in their classic volume, *The Power of Movement in Plants*². Although there were several early attempts to determine the spectral sensitivity of this response, it was Johnston who produced the first detailed action spectrum in 1934³. This study covered only the blue and green regions of the visible spectrum, showing a couple of peaks in the blue and a sharp cutoff in activity above 500 nm. On finding that oat coleoptile tips, classic objects for the study of phototropism, contained β -carotene, and noting the similarity of Johnston's action spectrum with the absorption spectrum of this common carotenoid, Wald and DuBuy⁴ concluded that the photoreceptor for phototropism must also be a carotenoid.

The picture became more complicated when it was shown^{5,6} that there was also a single broad band of activity in the phototropism action spectrum in the near-ultraviolet, where β -carotene does not absorb. At that time, a flavin or flavoprotein became the favoured photoreceptor, as flavins (at least in their oxidized forms) have the requisite absorption properties in the near-ultraviolet as well as in the blue region of the spectrum. This notion was strengthened by experiments demonstrating riboflavin-mediated photoinactivation of the plant growth hormone auxin⁷: differential flavin-mediated auxin destruction between illuminated and shaded sides of a growing plant organ could easily result in phototropic curvature — here was not only a viable photoreceptor pigment candidate, but a mechanism as well. This hypothesis was weakened by the observation⁸ that, at least in coleoptiles, there is no net change in auxin concentration during curvature; also, an auxin differential between light and shaded sides — described years earlier⁹ — requires physical continuity between the two sides of the organ. These results are consistent

with a mechanism of light-induced lateral transport of auxin away from the lighted side of the coleoptile, but wholly inconsistent with a mechanism involving auxin inactivation.

Another potential mechanism was proposed when it was shown that blue light could induce the reduction of a *b*-type cytochrome in the plasma membrane of plant cells both *in vivo* and *in vitro*¹⁰. This reaction is thought to be driven by a photoexcited flavoprotein. Could light-induced electron transport at or across the plasma membrane play a part in any of the various blue-light responses? Unfortunately, after almost two decades there is still no firm evidence, either correlative or genetic, to indicate whether this interesting photochemistry has any significant physiological function.

Most recently, pterins have been implicated as possible photoreceptors in several blue-light-sensitive systems¹¹, adding another candidate to the list of potential chromophores for blue-light perception. The suggestion was based largely on action spectroscopy and other evidence from fungi, unicellular algae and photosynthetic bacteria, but some of the arguments could be applied as well to higher plants.

A genetic investigation¹² of the regulation of plant growth by light using the model plant *Arabidopsis thaliana* resulted in the isolation of mutants altered in light-mediated hypocotyl growth inhibition, including the first blue-light-response mutant of a higher plant. Ahmad and Cashmore¹ have isolated several alleles of this blue-light-response mutant, one of which is tagged by a T-DNA insert, allowing the application of molecular genetics to isolate the wild-type gene. The tagged gene turned out to have homology with a whole class of prokaryotic proteins — the DNA photolyases — that mediate the repair of thymidine dimers induced

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by ultraviolet-B light in a blue-light-dependent manner¹³. The DNA photolyases operate through two functional chromophore moieties, one a reduced flavin, and the other either a deazaflavin or a pterin. In view of the gene's homology to light-activated flavo/pteroprotein enzymes and the wavelength dependence of the mutant phenotype, the authors suggest that this gene may encode the apoprotein of a blue-light photoreceptor, although definitive proof is still needed.

It is unlikely, should this molecule turn out to be the photoreceptor, that it operates through DNA-lyase activity. First, normal photomorphogenesis is not likely to be mediated by a photoactivated enzyme whose sole function is to repair damaged DNA. Second, the sequence lacks a critical tryptophan that is conserved in all authentic DNA lyases and is thought to be involved in binding the

enzyme to the thymidine dimer in damaged DNA. Third, it has a carboxy-terminal domain with modest homology to tropomyosin, a domain that is unknown in the photolyases but which might be important for some sort of protein-protein interaction.

It will be of great interest to learn the function of this intriguing protein and of any other related members of the same gene family in the light-regulation of plant growth. The study of Ahmad and Cashmore raises hopes that at last we may begin to learn about the molecular events in at least one signal transduction chain in higher plants that is activated by a blue-light photoreceptor. □

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NANOSTRUCTURES

Building regulations

Horia Metiu

TAKE a handful of atoms and toss them on to a flat single-crystal surface. They will stick there, then move, meet and aggregate. Do these aggregates prefer a specific shape? Is there some degree of order in the growth pattern? Can we control how this pattern looks by changing the growth conditions? We tend to think that orderly building is a human activity, requiring an architect and intelligent workers who follow instructions and check the results against the blueprints. Atoms move randomly, have no plan and are unable to cooperate. What they 'build' must look like the debris left over after an urban riot. Attempts to control their growth pattern would then seem pointless, as one could only change one chaotic mess into another. This view turns out to be wrong. As Kern and co-workers show on page 141 of this issue¹, adsorbed atoms form well organized aggregation patterns, whose structure can be manipulated experimentally.

To find the physical agent that creates order out of random motion we must examine the aggregation kinetics. The atoms change their position on the surface with a frequency controlled by the energy barrier between the starting and the final site. The magnitude of these barriers satisfies certain inequalities. Single-atom migration on the surface encounters the lowest barrier; the barrier preventing an atom from stepping down the edge of an 'island' of atoms on to the terrace below is larger; that controlling the motion of an atom along an island's edge comes next; and leaving an island is even slower. The difference between these energies is

reasonably large, so it is possible to find temperature windows for which some ato-



Ragged clusters and random growth may be the norm . . .

mic motions occur frequently and others are practically forbidden. This ordering in the magnitude of the barriers leads to self-organization; the existence of the temperature windows allows us to influence the resulting pattern.

To see how this works, let us start with the deposition of atoms on the surface. If the flux is low and the temperature is high, the atom landing on the surface can cover large distances before finding a partner; this means that pairs are formed while the surface coverage is low. Atoms deposited

later will join the existing pairs and the adsorbates will form a few large islands. The opposite conditions (high flux and low surface temperature) lead to a large number of small islands.

There is a temperature window in which the individual atoms are mobile but are unlikely to descend from an existing island before a second atom is deposited there. Islands are formed on top of islands and the growth is 'three-dimensional'; the atoms do not wet the surface. Wetting can be induced by working at higher temperature. We can use this to manipulate the growth process. For example, we can deposit less than a monolayer at high temperature to form a few large islands, then continue the deposition at low temperature to grow 'islands on islands' and form pyramids. Or we can first form a large number of small islands and then continue the deposition at high temperature to generate two-dimensional growth. The small islands can be created by low-temperature deposition², sputtering² or by use of surfactants (for example, antimony for silver deposition on the (111) surface of silver; ref. 3) to limit the mobility^{2,4} of the adsorbates.

Another temperature window controls island shapes. A complicated fractal-like structure^{1,5} is observed when the tempera-

ture is set to allow single-atom mobility but prevent the motion of atoms around the island's edge. This shape results because the atoms stick where they first made contact with the island. If such a fractal island is heated up, to allow atomic motion around the edge, its shape becomes rounded. At even higher temperature, the island will take its equilibrium shape with straight edges along the symmetry directions of the surface. In both homo- and heteroepitaxy on a (111) metal surface the equilibrium shapes are nearly regular hexagons^{1,5}.



. . . but copper atoms fall into line on this anisotropic crystal surface (scanning tunnelling microscopy by Röder *et al.*⁴).

In contrast, copper atoms deposited on a Pd(110) surface form myriads of long, one-atom-wide wires in the [110] direction¹, and silicon deposited on a Si(100) surface forms two-atom-wide wires^{6,7}. In both cases heating makes the wires thicker. These flawless, thinnest wires must have unusual transport properties, which the theorists may begin to explore. Unfortunately, before getting excited the experimentalist must figure out how to make electrical contact with them.

One of the most unexpected observations is that under certain conditions^{5,8} the atoms form, with very few errors, islands having precise shapes. A most striking example is provided by the deposition of platinum on Pt(111)⁵. If deposited at 425 K the atoms form equilateral triangles; at 450 K nearly regular hexagons are preferred; at 550 K the system reverts to forming triangles, but with a different orientation. The equilibrium shape, which is hexagonal, is reached at 700 K. This too can be rationalized⁸ in terms of a hierarchy in the energy barriers, but the story is too complicated to be told here.

Finally, high temperatures also make possible the exchange of atoms between islands. This will start yet another process: the coarsening of the islands. Small islands tend to disappear and the island size distribution becomes much more uniform¹.

Where does this leave us? We are learning to accept that crystal growth resembles a construction site more than a riot. Perfectly random motion can lead to self-organization. The order in the probabilities of different random jumps translates into geometric order in the resulting aggregates, whose shapes and length scales can be influenced experimentally.

This general scheme can have endless particular implementations. There is a chance that the difference between the face of a Hollywood starlet and that of the next-door neighbour is due to a few kilocalories difference in the right energy barriers. □

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A question of reduction

Chris Ballhaus

OXYGEN, the breath of life to us, also plays a vital role in the Earth's upper mantle. Although the fugacity of free oxygen in the mantle will rarely exceed 10^{-7} bar, it nevertheless determines whether the mantle contains a free fluid phase and whether this fluid will be rich in methane or carbon dioxide. Variations in volatile speciation affect the distribution of partial melt by altering the solidus temperature of mantle material¹⁻³, and this in turn influences the geophysical and rheological properties of the mantle. So the news that part of the upper mantle may be strongly reduced⁴ will be food for thought to petrologists, seismologists and mantle dynamics modellers alike.

The redox stratigraphy of the mantle and factors governing oxygen fugacity have been something of a mystery¹⁻⁵. In theory, several different redox equilibria in the mantle could buffer the oxygen fugacity: volatile equilibria such as $C + O_2 \rightarrow CO_2$ or $C + 2H_2O \rightarrow CH_4 + O_2$, ferric-ferrous iron equilibria such as $3Fe_2SiO_4 + O_2 \rightarrow 2Fe_3O_4 + 3SiO_2$, equilibria involving sulphide phases or carbonates plus elemental carbon, or any combination thereof. In practice, one or more of the endmembers in these equilibria is present only in dilute solid solution in crystalline mantle phases or (like the C-H-O volatiles) dissolved in a multi-component volatile or a melt. As a result, the oxygen fugacity becomes dependent on the bulk chemical composition (the bulk ratio Fe_2O_3/FeO) and bulk mineralogy (the chemical potentials of the ferric iron-bearing components or of carbon dioxide). For shallow peridotitic mantle, accessible to the petrologist through mantle xenoliths in basalts, fugacity ranges may be readily calculated from the composition, but not so for the deeper mantle.

Mineral synthesis

This is where O'Neill and co-workers⁴ step in. They have synthesized mineral assemblages that are believed to represent the mineralogy of the 'transition zone', 410 to 660 km deep in the mantle. They synthesized these phases (β -(Mg,Fe)₂SiO₄, known as β -phase, γ -(Mg,Fe)₂SiO₄, or γ -spinel, and majorite garnet) under extremely reduced conditions in the presence of stishovite (SiO₂) and metallic iron, deriving the oxygen fugacity from the reaction $Fe_2SiO_4 \rightarrow 2Fe + SiO_2 + O_2$ and the chemical potential of the Fe_2SiO_4 component in solid solution in the β -phase and γ -spinel. The fugacity thus recorded is about three orders of magnitude lower than the minimum calculated for peridotite xenoliths. Analysing

the synthesis products later by Mössbauer spectroscopy, they found that in all transition-zone phases much of the iron content was in the ferric form, and this despite the highly reduced conditions under which they were synthesized.

Obviously, this has important implications for the redox conditions in the transition zone. Ferric iron, which in the peridotitic upper mantle is concentrated in the minor phases spinel, garnet and pyroxene (the major phase olivine does not incorporate ferric iron), finds a vastly increased reservoir in the transition zone. As a result, the chemical potentials of the ferric iron-bearing components in solid solution will drop when transition-zone assemblages are stabilized, and so will the oxygen fugacity.

Implicit in this argument is that the bulk composition (and ferric iron content) of the transition zone is the same as that of fertile peridotitic mantle material (material capable of producing a basaltic melt)⁶, a notion that is not undisputed. Seismic evidence, in particular the sharpness of the 410-km discontinuity⁷ and the high velocity gradients within the transition zone, suggest that there may also be a chemical change at this discontinuity and that the upper mantle may not be as uniform in composition as we like to believe.

What of the redox structure of the overlying silicate upper mantle? Fertile peridotite undergoes a series of phase changes with increasing pressure, and these transitions must also affect the distribution and chemical potentials of the ferric iron components. The best documented reactions are the transitions from plagioclase to spinel (about 25 km down), and spinel to garnet (at about 90 km). A further increase in pressure will gradually push the aluminous and the ferric iron components from pyroxene into the garnet phase, until at around 9 GPa (200 km depth) the silicate endmembers of pyroxene become soluble in aluminous garnet⁸.

All these reactions tend to increase the mineralogical reservoir for ferric iron, albeit far more gradually than the phase transitions at the 410-km discontinuity. If we again assume that the bulk ferric iron content of the peridotitic mantle stays constant with depth — a reasonable assumption given the large-scale convective stirring of asthenospheric mantle — then the trend for reduction with depth will commence well within the peridotitic mantle, culminating with metal saturation in the transition zone⁹.

Then there are the implications for partial melting processes in the overlying

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silicate mantle. At such low oxygen fugacity, the volatiles present will be mostly the reduced species, methane and hydrogen, with minor amounts of water and virtually no carbon dioxide¹. Methane, so far as we know, does not react with mantle silicates, nor is it very soluble in melts (unlike CO₂), so the transition zone may be a reservoir where a fluid phase is indeed stable, as O'Neill *et al.* suggest. Under these conditions, 'redox melting' becomes a real possibility^{1,2}: where methane- and H₂-rich fluids infiltrate an overlying, gradually more oxidized silicate mantle, they will gradually become enriched in water and carbon dioxide until the H₂O and CO₂ activities are high enough for partial melting of the mantle to occur. Such redox fronts may explain the presence of a low-velocity zone¹⁰ within the peridotitic mantle, and a more focused fluid flow may initiate buoyant plumes in the overlying peridotitic mantle and ultimately cause intra-plate volcanism¹.

Recycling revisited

And finally, we may need to reconsider the effect of crustal recycling on the redox stratigraphy and isotope geochemistry of the upper mantle. For instance, it has been suggested that oxidized crust recycled into the convecting mantle may cause long-term oxidation of the mantle¹¹; that some basalts owe their oxidized nature to a crustally derived component that re-emerges in the course of large-scale mantle stirring¹²; and that the crustal input can be quantified on the basis of oxygen isotopes in basalts¹³. But if O'Neill and colleagues are right, any material recycled into the deeper mantle must pass through the transition zone and suffer reduction; moreover, its oxygen isotope signature would be swamped beyond recognition on encountering the methane-rich fluid phase. How realistic are any of our favoured models? □

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The invertebrate enigma

Roger C. Hardie

It is now almost ten years since excised patches of light-sensitive channels from vertebrate retinal rods were shown to be activated by cyclic GMP, effectively settling years of controversy over whether Ca²⁺ or cGMP was the final messenger of excitation¹. But how does the rest of the animal kingdom transduce light? This is one of the outstanding questions in sensory physiology, and was the topic for debate at a meeting last month*. Ironically, Ca²⁺ and cGMP are now two of the main candidates for the role of final messenger in invertebrate photoreceptors, but the underlying transduction machinery appears to be quite distinct from that operating in vertebrates.

Abundant evidence now implicates the phosphoinositide cascade in invertebrate phototransduction^{2,3}. This ubiquitous signalling system, characterized by production of inositol 1,4,5-trisphosphate (InsP₃), release of Ca²⁺ from intracellular stores and Ca²⁺ influx, is found in all manner of hormonally and neurotransmitter-stimulated cells.

The simplest current hypothesis — that Ca²⁺ released from InsP₃-sensitive stores activates the light-sensitive channels — now appears to be insufficient. Careful measurements using Ca²⁺ indicator dyes in both *Limulus* ventral photoreceptors

(H. Stieve, RWTH, Aachen; R. Payne, University of Maryland) and the drone bee (B. Walz, University of Regensburg), showed that the electrical response precedes the first detectable rise in Ca²⁺ by several milliseconds. As Payne wryly commented, this is not encouraging for a hypothesis that suggests that the rise in Ca²⁺ is causal for the light response. Furthermore, depletion of intracellular stores with a Ca²⁺ ionophore abolishes the light-induced rise in Ca²⁺ but leaves a large electrical response intact.

These experiments must be viewed in the context of extensive molecular, biochemical and pharmacological evidence for the direct role of both InsP₃ production and Ca²⁺ in excitation^{2,3}. One resolution to this paradox is suggested by evidence for the existence of as many as three parallel transduction pathways in *Limulus*: the phosphoinositide pathway, and others involving cyclic nucleotide metabolism (K. Nagy, RWTH, Aachen). Despite a conspicuous lack of evidence for the requisite biochemical machinery, this hypothesis has its roots in work showing that cGMP can activate channels in *Limulus*, even in excised patches⁴. A further indication of multiple pathways was provided by the ability of InsP₃, Ca²⁺ and cGMP to activate channels in excised patches of molluscan microvillar membrane (E. Nasi, Boston University). But the conclusion that all, or indeed

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Bread and water



"For in the wilderness shall waters break out, and streams in the desert." When Isaiah spoke of the joys that awaited Zion, he didn't anticipate the fate of the occupants of this bread lorry, who had to be winched to safety by helicopter. Flash floods such as this one in Israel's Negev Desert do not just temporarily foul up road communications: on page 148 I. Reid *et al.* report that desert rivers transport very much more coarse sediment than perennial rivers — mainly because they do not have the opportunity to build up the protective layer that covers the beds of their more established counterparts. If climate change causes an increase in the frequency of flash floods in dry regions, excessive sedimentation could be an additional headache for the locals.

G.W.

any, of these messengers directly gate ion channels is tempered by the physiological complexity of these patches, most of which still respond to light and so must retain elements of the entire transduction cascade. In an alternative approach, a cGMP binding protein was partially purified from squid photoreceptor microvillar membrane and incorporated into artificial liposomes loaded with Ca^{2+} . Cyclic GMP was shown to induce Ca^{2+} fluxes even in this reconstituted system (J. Brown, Yeshiva University, New York).

In *Drosophila*, there is a strong candidate for at least one light-sensitive channel: the *trp* gene, which encodes a putative Ca^{2+} channel subunit that has a calmodulin binding site but no consensus cGMP binding domain^{5,6}. The light response in *trp* mutants shows an altered reversal potential indicating reduced permeability to Ca^{2+} , and direct measurements, using Ca^{2+} -selective microelectrodes, show that light-induced Ca^{2+} influx is drastically reduced (B. Minke, Hebrew University, Jerusalem). Excitation in *Drosophila*, at least, is absolutely dependent on phospholipase C activity, suggesting that phototransduction might be an example of the poorly understood process of phosphoinositide-mediated Ca^{2+} influx ('capacitative Ca^{2+} entry') found in hormonally stimulated cells⁷. Cytosolic Ca^{2+} seems an unlikely final messenger of excitation in *Drosophila*. For example, photolytic release of caged Ca^{2+} fails to activate any channels in the plasma membrane, although it induces an electrogenic Na/Ca^{2+} exchange current and facilitates and inactivates light-sensitive channels with a submillisecond latency once they are already activated (R.C.H.).

Ca^{2+} is clearly of crucial importance for both excitation and adaptation; whether or not it gates the light-sensitive channels, there are many other potential sites of action. One candidate, calmodulin, seems to be transported into *Drosophila* microvilli by the *ninaC* gene product — a novel myosin-like protein (J. Porter *et al.*, Johns Hopkins University). Elimination of the calmodulin binding site in *ninaC* greatly reduces microvillar calmodulin and modifies the cell's electrical response. In addition, Ca^{2+} -induced Ca^{2+} release has now been demonstrated in an invertebrate (drone-bee) photoreceptor, and found to be caffeine- and ryanodine-sensitive as in other cells (Walz and O. Baumann).

Vertebrate and invertebrate photoreceptors have apparently adopted quite distinct signal transduction cascades,

although traces of a common evolutionary past may yet be uncovered in both⁸. Intriguing parallels, however, are found with the chemical senses — probably the first sensory modalities to evolve. Strong evidence also points to the direct involvement of phosphoinositide signalling in invertebrate chemical receptors (K. Ramming and H. Breer, University of Stuttgart). However, sensory cilia contain no Ca^{2+} stores and excised odour-sensitive channels from lobster olfactory sensilla can be directly activated by InsP_3 and even InsP_4 (H. Hatt, Ruhr University, Bochum; B. Ache, University of Florida). Alternative pathways also seem to exist in olfactory

cells, and cation-selective channels activated by both Ca^{2+} and 5'-GMP, along with a channel apparently gated by both protein-kinase-C-dependent phosphorylation and cGMP, were described (Hatt; F. Zufall, Yale University; M. Stengl, University of Regensburg). As in vertebrate rods, these results emphasize the importance of studying channels; further characterization of the light-sensitive channels may help to unravel the enigma of invertebrate phototransduction. □

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SIGNAL TRANSDUCTION

Cytokine connections

Tony Hunter

Two weeks ago, these pages carried a celebration¹ of the progress that has been made in working out the molecular details of the Ras-dependent signalling pathway which runs from the cell membrane to the nucleus. But great strides are being made in untangling another signal-transduction pathway which starts with the binding of interferons (IFNs) and other cytokines to their cell-surface receptors. The latest information as to the mechanisms concerned is to be found on pages 129 and 166 of this issue^{2,3}, where Kerr and colleagues describe their investigations into the pathways activated by IFN- α and IFN- γ .

Unlike the Ras pathway, the end result of which is the translocation of activated protein kinases into the nucleus where they phosphorylate and activate nuclear transcription factors, IFNs induce tyrosine phosphorylation and activation of transcription-factor subunits in the cytoplasm, which then translocate into the nucleus and induce transcription⁴. The initial insight into this other pathway was the finding that subunits of an IFN- α -induced transcription factor, ISGF3, are localized in the cytoplasm before IFN- α treatment^{5,6}. Following treatment, three of these cytoplasmic proteins (p113, p91 and p84) become phosphorylated on tyrosine, migrate into the nucleus, and assemble into a complex together with p48, a sequence-specific DNA-binding protein whose affinity for IFN- α -response elements (ISRE) in IFN- α -induced genes is thereby increased⁷⁻⁹. Molecular cloning of the ISGF3 subunits revealed that p91 and p84 are generated from the same gene by alternative splicing, and that p113, although derived from a distinct gene, is related over its whole length to p91 (refs 10, 11). p48 contains an amino-terminal DNA-binding domain related in sequence to those of the transcription factors IRF-1, IRF-2 and cMyb, and a carboxy-terminal

domain needed for the binding of the other ISGF3 subunits^{12,13}.

The next steps were made by analysing mutants of an HT1080 human fibrosarcoma cell clone, which expresses the bacterial *gpt* gene under the control of an IFN- α and IFN- γ -inducible promoter, selected for nonresponsiveness to IFN- α (ref. 14) (see table). Mutant complementation group U3 cells lack a functional p91 gene, and therefore express neither p91 nor p84. IFN- α responsiveness is restored to U3 cells by expression of p91 or p84 complementary DNAs (ref. 15). Mutant group U2 cells lack a functional p48 gene, and responsiveness is restored by expression of p48. Mutant group U1 cells, which contain functional p113, p91/84 and p48 genes and bind IFN- α , albeit at a somewhat reduced level, proved to be mutant in the TYK2 protein-tyrosine kinase (PTK) gene¹⁶. Up to that time TYK2 and the related nonreceptor PTKs JAK1 and JAK2 had no known function^{17,18}, but this finding suggested that binding of IFN- α to its receptor, which lacks intrinsic PTK activity, might lead to activation of TYK2, which in turn could phosphorylate p113, p91 and p84.

This raised the issues of how the IFN- α receptor would activate TYK2, and how tyrosine phosphorylation of p113, p91 and p84 would result in their translocation to the nucleus and association with p48. The presence in p113, p91 and p84 of an SH2 (Src homology) domain, which binds to phosphotyrosine in a defined sequence context in other proteins, provided one clue⁸. In principle, the SH2 domains of these ISGF3 subunits could bind to phosphotyrosine in TYK2 itself and/or to phosphotyrosine on one of the other subunits. A single tyrosine, Tyr 701, downstream of the SH2 domain is phosphorylated in p91 (ref. 19). A Tyr 701→Phe mutant form of p91 fails to restore IFN- α responsiveness

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RÉSUMÉ

Potent plastic

DON'T rely on the innocence of your laboratory plasticware. H. Glossmann and colleagues noticed a loss of biological responsiveness in samples that they were storing for activity analysis of membrane L-type calcium channels, and have tracked down the culprit (*Proc. natn. Acad. Sci. U.S.A.* **90**, 9523–9527; 1993). It turns out to be Tinuvin 770 — bis(2,2,6,6-tetramethyl-4-piperidyl) sebacate — a light stabilizer for polyolefins which leaches from plastic tubes. The compound proves to be pharmacologically intriguing. It selectively binds to the benzothiazepine site on the L-channel, a key site in the action of some cardiovascular drugs, and inhibits channel activity as much as the most potent agents known. But Ciba-Geigy, whose patent on Tinuvin 770 expired a couple of years ago, has no plans to exploit the compound therapeutically.

Killer trees

ASTEROID impacts may have done for the dinosaurs. But what caused the two far earlier mass extinctions of tiny marine animals during the Devonian period, more than 350 million years ago? At the annual meeting of the Geological Society of America last month, T. Algeo (University of Cincinnati) suggested that the first wave of destruction came about when tree-sized land plants became abundant, their huge root systems breaking up rock and leading to weathering. Nutrient-rich sediment washed out to sea — and there are signs of this in the strontium isotope signatures of the Devonian shales — could have fertilized giant algal blooms which robbed the water of oxygen. The second extinction coincides with the evolution of seeds, spreading the greenery still further afield.

Head starts

WHILE studying the role of catenins in cell adhesion mediated by cadherins, P. D. McCreia *et al.* (*J. Cell Biol.* **123**, 477–484; 1993) came up with an unexpected observation in the best traditions of developmental biology. The authors blocked the action of β -catenin in the ventral blastomeres of a four-cell *Xenopus* embryo by anti-catenin antibodies. But instead of the cell adhesion defect they expected, a new secondary body axis was induced. The most striking manifestation of this was two-headed embryos with complete duplication of external and internal head structures. The catenins therefore can be added to a select group of proteins of diverse structure and function that can elicit this phenotype — as McCreia and colleagues point out, however, catenins are unlikely to be the morphogens that provide the primary patterning information.

Components of the interferon- α and - γ signalling pathways

Protein	IFN- α response	IFN- γ response	Mutant cell line
p48	Required (DNA-binding subunit)	Not required	U2 (IFN- α^- , IFN- γ^+ , p48 $^-$)
p84	Required/ phosphorylated	Not required	U3 (IFN- α^- , IFN- γ^- , p84/p91 $^-$)
p91	Required/ phosphorylated	Required/ phosphorylated (DNA-binding subunit)	U3 (IFN- α^- , IFN- γ^- , p84/p91 $^-$)
p113	Required?/ phosphorylated	Not required	No mutant known
JAK1	Required/ phosphorylated	Required/ phosphorylated	U4 (IFN- α^- , IFN- γ^- , JAK1 $^-$)
JAK2	Not required	Required/ phosphorylated	γ -1 (IFN- α^+ , IFN- γ^- , JAK2 $^-$)
TYK2	Required/ phosphorylated	Not required	U1 (IFN- α^- , IFN- γ^+ , TYK2 $^-$)

to U3 cells and is not translocated to the nucleus, establishing the importance of this tyrosine phosphorylation in ISGF3 activation¹⁹.

IFN- γ has an unrelated receptor and induces expression of a different but overlapping set of genes to IFN- α through a distinct response element known as GAS (refs 2, 20). IFN- γ stimulates tyrosine phosphorylation of cytoplasmic p91, but not of p113 or p84, and phosphorylated p91 binds directly to the GAS element after translocation to the nucleus^{21,22}. IFN- γ -induced tyrosine phosphorylation of p91 also occurs at Tyr 701 and this modification is required for GAS binding¹⁹. The failure of U3 cells to respond to IFN- γ is consistent with an essential role for p91 in the IFN- γ response. IFN- γ responsiveness is restored to U3 cells by expression of p91, but not p84. The selective phosphorylation of p91 in response to IFN- γ suggests that a PTK distinct from TYK2 is responsible.

Kerr and co-workers now shed some light on the nature of this PTK. In the first paper² they show that the U4A mutant line, which responds to neither IFN- α nor IFN- γ , expresses a truncated form of *JAK1* messenger RNA and no JAK1 protein, and can be complemented for both IFN- α and IFN- γ responses by expression of JAK1 cDNA. In parental cells IFN- α induces tyrosine phosphorylation of both TYK2 and JAK1, but does not induce phosphorylation of either JAK PTK in U4A cells. Similarly, in U1 cells IFN- α fails to induce either JAK1 or TYK2 phosphorylation.

In the second study³, the group selected a new HT1080 cell mutant, γ 1A, unable to respond to IFN- γ . However, γ 1A cells still respond to IFN- α , and have functional p113, p91/p84 and p48 genes. IFN- γ stimulates both JAK1 and JAK2 tyrosine

phosphorylation in parental cells, but neither JAK1 nor JAK2 is phosphorylated in γ 1A cells. Expression of a JAK2 cDNA in γ 1A cells restores IFN- γ responsiveness, and IFN- γ -induced phosphorylation of JAK1 and JAK2. A full length JAK2 protein is detected in γ 1A cells, so it seems likely that there is an inactivating point mutation in JAK2 in γ 1A cells (although the mutation could conceivably be in another protein required for activation of JAK2, which is bypassed when JAK2 is overexpressed).

Activation of JAK family PTKs by IFN- γ and IFN- α can be summarized as follows (see table). IFN- γ elicits tyrosine phosphorylation of JAK1 and JAK2, but not TYK2, whereas IFN- α induces phosphorylation of JAK1 and TYK2, but not JAK2. Both U1 and U4A cells fail to respond to IFN- α and lack functional TYK2 and JAK1 respectively, which implies that both of these JAK PTKs are needed for the IFN- α response. Likewise, the failure of U4A and γ 1A cells to respond to IFN- γ indicates that both JAK1 and JAK2 are required for the IFN- γ response. The other conclusion that can be drawn from the patterns of JAK PTK activation in these mutants is that the JAK PTKs cannot be placed in a linear order where one JAK PTK activates another, because if either JAK PTK in a pair is inactivated the other is not phosphorylated. How then do the two JAK PTKs required in each case cooperate in a receptor-specific manner to elicit mutual activation? (Note, though, that little is known about JAK family PTKs, and it has yet to be shown that elevated tyrosine phosphorylation is reflected in increased activity towards substrates (although this is a reasonable surmise) or that they can phosphorylate p113 or p91/84 directly.)

A possible clue to the activation

mechanism comes from the demonstration that JAK2 associates with the erythropoietin and growth hormone receptors and is activated upon ligand binding^{23,24}. If the two JAK PTKs required were to interact simultaneously with the cytoplasmic domain of the relevant IFN receptor, this would facilitate mutual activation by transphosphorylation in a manner similar to that in which the two subunits of a ligand-bound receptor PTK dimer transactivate. JAK PTK activation might require the unusual protein kinase-like domain that lies to the amino-terminal side of the protein-tyrosine kinase catalytic domain in these proteins¹⁸. But the exact mechanism involved in JAK PTK activation may be more complicated, for a protein-tyrosine phosphatase may be involved²⁵.

A surprise in this system came in September with the finding that tyrosine phosphorylation and activation of p91 is not the preserve of the interferons, and that many cytokines and growth factors can induce tyrosine phosphorylation of p91 or p91-related proteins through response elements related to GAS²⁶⁻²⁹. But a JAK PTK may not be responsible for p91 phosphorylation in every case.

For instance, some of the cytokines, such as epidermal growth factor (EGF), that induce p91 phosphorylation directly activate receptor PTKs. Moreover, p91 binds to the activated EGF receptor, and both p91 binding and its ability to stimulate transcription depend on the integrity of its SH2 domain³⁰, suggesting that the EGF receptor itself phosphorylates p91. However, because such cytokines induce specific phosphorylation of Tyr 701, a JAK PTK may prove to be involved. Activation of p91 by receptor PTKs shows some specificity because the fibroblast growth factor receptor does not activate p91 (ref. 29). Whatever the mechanism of activation of p91 by growth factor receptor PTKs, this activation may be important for the induction of a subset of immediate early genes. For instance, it has been shown that the SIF (*sis*-inducible factor) response element in the *c-fos* gene, which has a core sequence related to the GAS element, binds tyrosine-phosphorylated p91 and that this activates transcription^{28,30}.

In a short time we have gone from what appeared to be a membrane-to-nucleus transduction pathway unique to the interferons to one that is almost universally activated by cytokines. The different cytokines that activate p91 do not all elicit identical cellular responses, so there must be as yet unidentified elements of specificity. This specificity may be determined by whether it is p91 or a p91-related protein that is activated, which in turn may depend on which JAK family PTK is activated, and on the binding specificity of the p91 family member SH2 domain.

There are three known PTKs of the JAK family, but there are likely to be other members that may cooperate with JAK1, JAK2 or TYK2 to phosphorylate individual p91-related proteins. If one assumes that one of the two cooperating JAK PTKs is simply required for activation of the other, then one could explain the observed patterns of phosphorylation of p113, p91 and p84, if JAK1 phosphorylates p91 and TYK2 phosphorylates p113 and p84. This, though, needs to be established biochemically.

It also has to be determined whether receptor PTKs can phosphorylate p91 directly, or whether they also require activation of a JAK PTK. In IFN- γ -treated cells, a p91 dimer binds to the GAS element. But other cytokines induce GAS-binding activities with different electrophoretic mobilities, which suggests that there are additional subunits present in these complexes. Ancillary subunits could alter the DNA sequence preference of p91 family members, and it is becoming clear that there are several GAS-related sequences that have different affinities for p91. Given that p113 is closely related to p91, it is possible that it too can be activated by tyrosine phosphorylation to bind to DNA in a sequence-specific fashion. □

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Plastic memories

If you warm a typical polystyrene food tub cautiously over a low flame, it may flatten completely, and revert to the disc from which it was formed. This is 'plastic memory'. Many polymers show it, and even some metals. The Nitinol alloys can be hot-formed, and then cooled to freeze in the deformation. On subsequent heating, they snap back to their original shape with some force. Daedalus once designed a car made of memory plastics and Nitinol alloys. If it got dented in a collision, the owner merely warmed up the relevant area with a hot-air blower, when it snapped back to its original shape. He now sees the phenomenon as the basis for a novel system of product recycling.

Many plastics objects carry labels claiming that they can be recycled. However, almost nobody is foolish enough to try it: the resulting polymeric mixture is practically useless. At the moment, recyclability is merely an expensive massage for tender middle-class consciences. And yet, says Daedalus, many margarine tubs, yoghurt pots, beverage bottles and other guilt-inducing plastic packages, could be recycled very directly by exploiting their in-built plastic memory. Simple controlled heating could snap them back to basic discs, from which other pots could then be pressed. They would not have to be melted back to bulk polymer.

So while DREADCO's chemists are perfecting a commercial memory-plastic, its engineers are designing a standard set of memory-plastic billets. A well-chosen basic repertoire of flat discs, rectangular slabs and hollow cylinders, should permit plastics formers and packaging companies to form an endless range of bottles, tubs, pots, boxes and so on. After their short life, these products could be collected *en masse*, cleaned, and warmed up. They would then snap back to basic billets, which could be easily and automatically sorted for return to the plastics formers.

Even quite complex consumer products could be designed for memory-effect recycling. Telephones, radios, electric mixers and so on, generally consist of a central 'works' encased in a plastic shell. This could easily be formed from one or more billets, held together by Nitinol alloy rivets. On heating the object above the transition temperature, the rivets would relax, letting the shell flatten back to its original billets. The product could be reformed in the latest style, and sold again. All the delights of repeated cosmetic package updating and constant trivial product-churning could be reconciled with ecological, planet-saving rectitude.

David Jones

Bright flash on Jupiter in 1983

SIR — Predictions that Comet Shoemaker–Levy 9 will collide with Jupiter in July 1994 (refs 1, 2) have spurred interest in the observable consequences of the impact^{3,4}. The collision is predicted to occur on the far side of Jupiter, thus the most likely instantaneous observable effect will be a flash that momentarily illuminates a satellite located behind Jupiter at that time. We present evidence

— in addition to Io. In all frames (up to, including, and following the frame that showed Io as bright) values for the sky and Europa were nominal, indicating that there was no problem with the frame itself. Io was unresolved, but its point-spread function subtended over 12 pixels. All pixels containing Io flux were bright, thus excluding photometry contaminated by a 'hot' pixel. In a charge-coupled device image, a cosmic-ray strike directly on Io could mimic a flash, but Vidicon detectors are not susceptible to such events. Although we cannot exclude a peculiar instrumental effect involving only the pixels containing Io in this frame, this explanation seems unlikely.

Could Io itself have suddenly had a brief intrinsic increase in brightness? Volcanic events are not likely candidates because of the strict constraint on the duration to 118 s. A specular reflection of a large smooth area might cause a brief flash, but such an effect has not been seen before on Io's surface, even at very high spatial resolution in the Voyager data. Io's atmosphere is not thick enough to create a fireball due to a striking body, nor has it shown any evidence for lightning, let alone lightning of this magnitude. Thus an event associated with Io itself is an unlikely explanation for the observed

anomalous brightening.

A flash on the far side of Jupiter could have manifested itself as an increase in Io's brightness. One plausible phenomenon is a 'superbolt' of jovian lightning⁷. Alternatively, calculations of the effects of Shoemaker–Levy 9's impact predict a flash as the comet plunges into the jovian atmosphere. The 50 per cent increase seen here, corresponding to an energy of about 10^{28} ergs, would be comparable to a 5-km icy body (with a density of 1 g cm^{-3}) with a velocity of 60 km s^{-1} converting its kinetic energy to luminous energy in 10 s with an efficiency of 0.1. More sophisticated power and energy dissipation estimates for typical cometary bodies predict flashes that would increase a satellite's brightness by 10–30 per cent for a brief interval (less than tens of seconds)^{3,4}. If the flash was caused by an impact, either the body was somewhat larger or faster than a typical comet, or else the luminous efficiency for

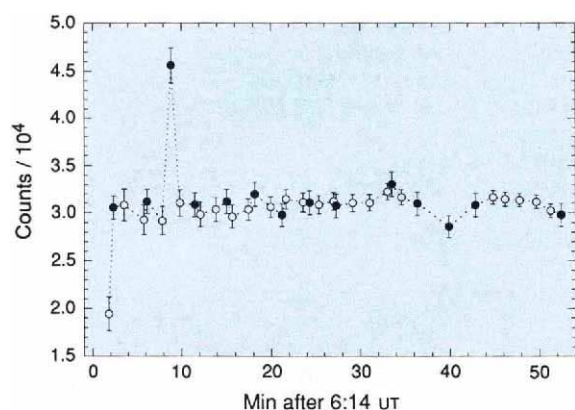
converting energy to brightness — which is not well known for Jupiter — may be somewhat larger than anticipated. An impact could also have had other observable effects on the jovian atmosphere; we encourage investigators with long-term datasets to check Jupiter during this time period to search for such phenomena.

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Brightness of Io on 26 July 1983 as it reappeared from Jupiter eclipse. The brightening at 6:22:50 UT (8.8 min after 6:14 UT) may be evidence for a flash on the far side of Jupiter. Closed circles, data (obtained with the Palomar 1.52-m telescope through a 4,200-Å filter) from Vidicon images that also included sky background and Europa, which was in front of Jupiter at the time of the observations⁵. The sky and Europa showed no unusual brightening in the frame in which Io was anomalously bright; all pixels containing Io in that frame were 50 per cent higher than the preceding and following frames⁶. Open circles, data (taken with a 0.61-m telescope on Mauna Kea through a 4,400-Å filter) from disk-integrated photometry⁷, showing no evidence for a flash, constraining the duration of the event to less than 118 s. The data in ref. 6 were normalized to match the data in ref. 5 in the 50 min after 6:14 UT (excluding the anomalous datum in ref. 6 and the first datum in ref. 5 when Io was still in eclipse; eclipse ended at 6:17 UT).

that such a flash on Jupiter's far side may have been detected in 1983.

On 26 July 1983, we were involved in collecting observations of the jovian moon Io as it reappeared from jovian eclipse^{5,6}. R. M. Nelson *et al.*⁶ took a series of 10-s integrations; one of these data showed an anomalous 50 per cent brightening of Io roughly 6 min after reappearance in one frame (see figure). Several 3-s integrations by H. B. Hammel *et al.*⁵ bracketed the anomalous flash, but did not overlap it; they showed no brightening, thus constraining the flash duration to be less than 118 s. Explanations for Io's anomalous brightening include: (1) instrumental effects in the data; (2) an event on Io itself; and (3) illumination of Io by a bright event on the far side of Jupiter.

The data in ref. 6 were Vidicon images containing sky background (scattered light from Jupiter) and the moon Europa — which was in front of Jupiter at the time

X larger than Y

SIR — The possibility of determining the sex of offspring in humans and animals is a topic of enduring interest. In 1921 it was found that haploid sperm carry either the X or Y chromosome¹, and that after fertilization with the haploid oocyte determine the sex of the offspring. Until now no scientific data have been reported concerning the size differences between X and Y sperm in humans and animals. We have used polymerase chain reaction (PCR) amplification with primers from the human Y-linked testis-determining gene (SRY; ref. 2) on human blood DNA from 41 male and female subjects. We identified that the primers are suitable for distinguishing between human male and female DNA. We then used the same primers (together with common control primers from the human sperm receptor gene ZP3 on chromosome 7; ref. 3) to identify 20 (male and female) single lymphocytes. Our results were 100% correct.

The same primers were used to determine the sex of 233 non-motile sperm (11 donors), which were individually selected for direct photography before PCR amplification. Two hundred and seventeen sperm (93.1%) showed satisfactory amplification, 106 (48.8%) being Y and 111 (51.2%) X. Under 'blind' conditions, we magnified photographs of individual sperm a further 30× and directly measured them. Statistical analysis showed the length, perimeter and area of the heads and the length of the neck and tail in X sperm are significantly larger than those of Y sperm. Thus, in contrast with earlier work^{4,5} our direct methods show for the first

time that human X sperm are statistically larger and longer than Y sperm.

Difference in size is one of the basic characteristics (such as speed, weight and so on) of X and Y sperm. It would be useful to analyse further these basic characteristics to develop sex-selected insemination programmes. The ability to correlate the sperm gene and chromosome condition with individual sperm shape and size also allows further investigation of the relationship between the morphological characteristics of individual sperm and some genetic diseases, which may improve the current procedures of preimplantation diagnosis by selected insemination or single sperm injection.

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■ A more detailed version of this manuscript is to be submitted for publication elsewhere.

Extinction rate estimates

SIR — Smith *et al.* in Scientific Correspondence¹ propose a new approach to estimating species extinction rates. They use International Union for Conservation of Nature and Natural Resources (IUCN) conservation categories, and look at the changes in 'Red Lists' for threatened animals (published at 2-year intervals) and in the World Conservation Monitoring Centre plant database to assess extinction rates. Central to their approach is the issue of how species are included in Red Lists and assigned categories. As already pointed out by Smith *et al.*, definitions of IUCN conservation categories are "largely subjective, and as a result, categorization made by different authorities differ and may not accurately reflect actual extinction risks"². Similarly, Red Lists are biased towards attractive, spectacular and (sometimes) better-known species. Most of these lists underestimate the number of species at risk.

To be useful, the system proposed by Smith *et al.* would have to be based on species conservation status categories obtained in an objective way, which relate to the probability of extinction. Then species status trends could be evaluated through time. Current IUCN categories do not fulfil these criteria and cannot be

used to obtain even a rough meaningful estimate of extinction rates. This is unfortunate because, to my knowledge, IUCN Red Lists are the only data of this type generally available for different plant and animal groups. (A more objective system is being sought, however³).

Changes in IUCN conservation category are related to existing knowledge about a given species, and not necessarily to real changes in probability of extinction. Some species have changed category, for example, simply because somebody looked for them in the wild. In such cases, what changed was the available information, rather than the actual status of the species. Further, Red Lists used are published at 2-year intervals, which reflect improvement in knowledge rather than a change in extinction risk (unless there has been a catastrophic or serendipitous event).

Smith *et al.* suggest that for higher vertebrates and palms their approach may reveal more about extinction rates than what is already known. But even for the better-known taxa the information in Red Lists is very limited and biased. Take, for instance, the case of Mexican mammals. Mexico ranks second in the world in the number of mammal species³, with approximately 450 land and 50 marine species⁴. Of land mammals, some 217 (48%) species are endemic to Mexico and Central America⁵, 149 (33%) are endemic to Mexico, and about 47 (10%) have very restricted ranges (<1,000 km²) and are known only from a few localities⁴. Many of these species are rare⁶ and are prone to extinction, especially given high habitat conversion rates. Hence they should be included at least in the 'rare' or 'indeterminate' IUCN categories, but only 15 (one endemic)⁷, 22 (five endemic)⁸ and 34 (fourteen endemic)⁹ Mexican land mammal species are in fact included in the Red Lists for 1986, 1988 and 1990. Other, more comprehensive, lists include 108 species (60 endemic)¹⁰ and 128 species (61

endemic)⁴, these lists together include 162 species (79 endemic). Diamond¹¹ has presented a similar case for the birds of the Solomon Islands.

Smith *et al.* argue that "estimates of extinction rates for the better-known taxa are of the same order of magnitude as those derived from totally unrelated species-area relations". But given the uncertainties, these estimates may not be correct. I agree with Smith *et al.*, however, on the urgency of more research on the dynamics of species extinction.

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What mercury pollution?

SIR — The importance of silver mining in South America over the past 400 years as a source of mercury in the environment has been discussed in Scientific Correspondence^{1,2}. Clearly, accurate analytical data are required for sensible discussion.

Camargo² makes the reasonable suggestion that the oceans are the principal reservoirs of mercury via inputs from the atmosphere and discharges from rivers. But by accepting the average concentration of mercury in unpolluted sea water as 0.1 µg l⁻¹, he overestimates the total quantity of mercury in the oceans by a factor of more than 100. The value of 0.1 µg l⁻¹ was accepted as typical in the 1970s, when techniques of analysis and control over contamination were not as well-developed as they are today. The concentration of mercury in coastal sea water is around 1 ng l⁻¹ or less, and the average concentration in the world's oceans is considerably less^{3,4}.

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Scientific Correspondence

Scientific Correspondence is a relatively informal section of *Nature* in which matters of general scientific interest, not necessarily those arising from papers appearing in *Nature*, are published. Because there is space to print only a small proportion of the letters received, priority is usually given according to general interest and topicality, to contributions of fewer than 500 words, and to contributions using simple language.

Worse than the malady

Roy Porter

Colonizing the Body: State Medicine and Epidemic Disease in Nineteenth Century India. By David Arnold. University of California Press: 1993. Pp. 354. \$45, £40 (hbk); \$18, £14.95 (pbk).

WE have had many gung-ho histories of colonial medicine, and it's sometimes argued today that medical progress forms the chief, if perhaps the only, enduring justification of imperialism. But a revisionist reading has arisen, contending that tropical medicine itself was just an arm of imperial power. In a superb survey, deeply researched and enviably lucid, David Arnold seeks a balanced verdict on European medicine's impact upon the 'jewel in the crown'.

British doctors in India at first operated within an 'enclavist' framework: white doctors would primarily tend white patients, essentially the officials and soldiers of the East India Company. Such circumstances lent themselves to a certain scholarly curiosity towards Ayurvedic and Yunani medicine (Hindu and Islamic), which were viewed as exotic and fascinating.

In time, however, the imperial mission inevitably expanded and the role of medicine grew with it. By the time of the accession of Queen Victoria, medical men were arguing that it was the duty of the British to bestow the blessings of medical science and public health on a land which (no one would deny) suffered appallingly from cholera, smallpox, dysentery and all the other 'diseases of warm climates' as they were then termed.

Yet the British medical establishment in India soon found itself in a cleft stick. It adopted a lofty opinion of its own superiority. Earlier sympathies towards native medicine evaporated: in Lord Macaulay's view, Ayurvedic medicine 'would disgrace an English farrier', and deserved to be supplanted. In so far as the British supported medical training for natives, it was to be in Western medicine, taught through the medium of English. It was, moreover, the Empire's duty to bring health to a backward, insanitary population. Yet resources were lacking to achieve this; as disdain grew for the indigenous population, the notion of spending precious millions on the health of expendable locals hardly commended itself to viceroys.

The Indian Medical Service also had to operate within the framework of *laissez faire*. Such economic doctrines frequently frustrated medical initiatives — for example, the imposition of quarantine to stop the spread of cholera. Health was a desideratum, but it must not hinder trade. And the ruling out in this way of certain

energetic interventions (can't do, won't do) in turn encouraged leanings in top circles towards epidemiological fatalism: the root cause of the prevalence of disease in the subcontinent, it was claimed, lay in the nature of the Indian climate, soil, people and mentality. Epidemics could no more be prevented than the monsoon. The continuing hold of cholera and en-

for their part, had developed immunity to tropical diseases.

Although Europeans were often sicker than the natives, and despite lack of assets, the itch to impose imperial medicine proved irresistible. Early in the nineteenth century, the death-rate of British soldiers (garrisoned in official barracks) was higher than that of Indian sepoy (who made their own living arrangements). Nevertheless, officials thundered against the dirt of the sepoys; in time they were made to live in barracks too — with the result that, by the end of the century, their health profile was no better than that of Europeans.

Another botched intervention was smallpox control. Indians had long had an indigenous tradition of smallpox variola-

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

The healing goddess Śītavātī, from a Buddhist manuscript of the *Pañcarakṣā* (first mentioned in the fourth century). Written in Sanskrit, the text invokes the goddess to combat snake bites, derangement and other ills, using prayers and spells.

teric fever among the natives may even have been psychologically reassuring: it confirmed convenient ethnocentric stereotypes of Asiatic ignorance, apathy and dirt.

Finally, the confident, aggressive medical mission was time and again undermined by the brute fact that, despite all the drum-banging, Western medicine didn't actually work. Faced with fevers, European medicine possessed no more panaceas than did Ayurvedic medicine.

This sad truth was cruelly underlined by morbidity and mortality statistics. Especially in early decades, Westerners in India died like flies, particularly soldiers. How could it be that the Anglo-Saxon race was sicker than squalid natives? Plausible explanations were, of course, to hand. Europeans were unused to the appalling heat and humidity; whereas, by habitually living in filth, the natives,

tion (associated with the goddess Sitala). The British medical establishment knew better: inoculation was unsafe, and must be replaced by vaccination. Campaigns were launched to prohibit inoculation and promote vaccination (and later make it compulsory). But they were bungled all down the line. Cowpox lymph had to be imported from the United Kingdom, which meant that much of it was useless. In deference to the sacred cow, there was powerful native resistance to vaccination. And the programme was always starved of funds. Wiser counsels in high places advised soft-peddalling with such programmes, lest they provoke another mutiny.

Bubonic plague produced the most comprehensive official response, and the fiercest resistance. With epidemics reaching horrifying proportions in the 1890s, the authorities assumed almost unlimited powers over the bodies of sick

and suspect Indians, riding roughshod over what were insensitively termed 'caste superstitions'. Indian opinion in turn united in opposition to the 'filthy habits' of Western medicine, including the mass public medical inspection of suspects and the searching of houses by soldiers. Imperial medicine thereby galvanized anti-imperial wrath.

Arnold is scrupulously fair and avoids being judgemental. On the evidence here adduced it is hard not to regard what he dubs 'the colonizing of the body' as at best largely ineffectual and at worst disastrous. Western medicine was all too often racially arrogant and culturally ignorant; in any case, it had few cures in the bag. Westminster was never prepared to invest the resources — they would have been colossal — to create a proper health administration. To a large degree, in consequence, medicine served principally as an ideological weapon for supporting white superiority. For the Indians, its pills were bitter. It probably created more wounds than it healed. □

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Light in motion

Richard N. Dixon

Photodissociation Dynamics. By Reinhard Schinke. Cambridge University Press: 1993. Pp. 417. £50, \$89.95.

THE past decade has seen a symbiosis of molecular spectroscopy, photochemistry and electronic structure theory that has revolutionized our knowledge of primary molecular photochemical processes. Experimental tools that have made this possible are tuneable pulsed dye lasers, optical harmonic generation of ultraviolet and vacuum ultraviolet light, and good cheap pulsed molecular beams. Fast computers with large memories can calculate with almost 'chemical accuracy' molecular potential energy surfaces for small molecules, with which classical or quantum trajectories can be run to simulate experiments. This new understanding of molecular dynamics has wide applications in photochemistry and the theory of chemical reactions.

This book provides a comprehensive theoretical description of nuclear motion

during photodissociation, and its relationship to observable phenomena such as molecular absorption spectra and the internal energy distribution within photofragments. A central theme concerns the various ways in which the multi-dimensional potential energy surface of a molecule can influence the outcome after its excitation. The author has adopted the policy of using a few well chosen molecules (H_2O , H_2O_2 , H_2S , ClNO and CH_3ONO) to illustrate the many dynamic features, rather than attempting a comprehensive experimental review. An exact description of the dynamics requires the use of quantum mechanics, but Schinke shows how most experiments can be understood at least qualitatively through classical mechanics.

The text intersperses a description of salient experimental findings with the theory necessary to explain them. The author starts with molecular absorption spectra, showing how any resonant structure arises from an excited complex that can live for at least a few vibrational periods before it breaks apart. He then proceeds through a general description of photodissociation to the more specific features of excitation of the photoproducts. He shows how their internal energy distribution reflects a mapping of the initial excitation onto the potential energy surface, and how this varies with the interatomic distances and angles as the fragments move apart. The book ends with theory on the experimental control of photodissociation, and with recent real-time experiments on the dynamics of photodissociation.

One of the strengths of the book is that it can be read at more than one level. A newcomer to the field could skip the mathematical details without losing the thread of the essentially physical description; bullet-points concisely summarize the most important features. The applications of time-independent quantum theory, time-dependent quantum theory and classical mechanics are all described in detail as appropriate, and interrelated wherever possible. Schinke has amply fulfilled his declared aim of providing an overview of the field for graduate students in molecular physics and for experimentalists who are not familiar with the quantum theory of photochemistry. □

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Autumn Books

Nature's next review supplement is Autumn Books, which will be published on 25 November. The issue will feature, among others, James Jasper on the atomic bomb, Rupert Hall on defining science and Roy Porter on gender in the making of science.



COLD snap — young adult king penguins, displaying to win their first mate, stand out in a creche of nearly full-grown chicks who have survived their first winter. This picture by Ben Osborne is taken from *Life in the Freezer* by Alastair Fothergill (BBC Books, £18.99), a natural history of the Antarctic, which is considered to be the last unspoilt continent. Full of clever and unusual colour photographs of wildlife adapted to the harshest of environments and of the scenery about them, it accompanies a BBC television series, presented by David Attenborough, to be released shortly.

Local flavours

R. D. Martin

Topics in Primatology. Volume 1: Human Origins. Edited by Toshisada Nishida, William C. McGrew, Peter Marler, Martin Pickford and Frans B. M. de Waal. Pp. 475. **Volume 2: Behavior, Ecology, and Conservation.** Edited by Naosuke Itoigawa, Yukimaru Sugiyama, Gene P. Sackett and Roger K. R. Thompson. Pp. 412. **Volume 3: Evolutionary Biology, Reproductive Endocrinology, and Virology.** Edited by Shozo Matano, Russell H. Tuttle, Hidemi Ishida and Morris Goodman. Pp. 489. Tokyo University Press: 1992. ¥25,000.

THE biennial congress of the International Primatological Society (IPS) is now a well-established event, typically attracting about 500 participants. This publication contains papers from 15 special symposia of the thirteenth congress, held in Kyoto (Japan) in July 1990. The proceedings of the congress itself were edited by A. Ehara *et al.* and published in *Primatology Today* (Elsevier, 1991).

The IPS has for some years held at least some congresses in countries inhabited by primates, thereby allowing participation of local primatologists. This local interest is one of these proceedings' strengths — most notable are the useful reviews of work on the Japanese macaque (especially social behaviour and reproductive endocrinology). Two useful contributions by L. Fedigan and P. Asquith and by V. Reynolds examine the historical significance of Japanese research into behaviour of the local macaques (*Macaca fuscata*).

Although Japanese studies reflect trends in primatology, they have also catalysed certain areas. Kinship was first recognized as an important influence on primate social life by Japanese primatologists, reflecting their training as anthropologists rather than biologists or psychologists (as in the Western tradition). Other prominent Japanese findings relate to male migration, matrilineal relationships, troop fission, inbreeding and gene flow. And the long-term nature of several Japanese studies allows analysis of life-history data spanning four decades. There have also been weaknesses: widespread provisioning of primates and limited response to the 'sociobiological revolution' (although some may regard the latter as a decided advantage).

Primatology is unusual in that practitioners are united by their animals (primates) rather than by any particular discipline. IPS congresses — and these proceedings — accordingly cover a wide spectrum. Volume 1 covers the origins of monogamy, the behaviour of chimpanzees and bonobos, the acoustical basis of

vocal communication, cognitive processes, molecular evolution of hominoids and hominid origins (neontology and palaeontology). Volume 2 deals with recent trends in studies of Japanese macaques, behaviour and stress, the concept of 'oestrus' in nonhuman primates, socioecology of gorillas and primate conservation. Volume 3 examines the phylogeny of prosimians, primate locomotion, origins of bipedalism, hormones and behaviour in primate reproduction and viral infections of nonhuman primates. Given the enormous scope of the material (in a total of 109 papers), this review can do no more than pick out a few highlights.

One well-covered topic is the socioecology of African great apes. Discussion of the behaviour of chimpanzees and bonobos confirms the striking and consistent differences between the two species. In captivity, during feeding or after conflict, bonobos show a marked increase in sexual behaviour, whereas chimpanzees emphasize kissing, embracing and touching (F. de Waal). Reviewing field studies, F. White and A. Lanjouw conclude that, in contrast to chimpanzees (which show female migration and male-centred groups), bonobos have a female-biased social system. R. Wrangham and colleagues report on a long-term study of female social relationships and social organization of chimpanzees in Kibale Forest (Uganda), checking an earlier report by M. Ghiglieri (1984) that this population shows a bonobo-like pattern with more female than male association. It emerges that the pattern of social organization in Kibale resembles that of common chimpanzees studied elsewhere, so the differences between chimpanzees and bonobos are consistent.

A particularly interesting contribution by J. Wallis follows up on her previous observations on synchronization of ovarian cycles in female chimpanzees housed together. Her analysis of data from Gombe Stream (1975–90) for initial postpartum swellings revealed significant cycle synchrony with a female com-

panion in cases where a focal female had lost an infant from the previous pregnancy, but not if the infant had been weaned. Further, social cueing occurred only if the companion female was fertile. There are also two useful reports on predatory behaviour of chimpanzees. In the first, S. Uehara and colleagues review a hundred cases of predation by chimpanzees in the Mahale Mountains National Park (Tanzania) for the period 1983–90, including comparison with data from other study areas (Gombe and Tai). It has consistently emerged that red colobus are the most frequent prey. Further, predation is predominantly (but not exclusively) a male activity. Overall, Mahale proves to be more like Gombe than Tai with respect to predation. In the second contribution, T. Nishida and colleagues report evidence for meat-sharing as a coalition strategy used by an alpha male chimpanzee.

Another key set of papers discusses the acceptability of the term 'oestrus' for describing sexual behaviour of female nonhuman primates. Contributors predictably argue for (T. Furuichi, R. Nadler, N. Okayasu) or against (A. Dixon, R. Goy) using the term for simian primates (monkeys and apes). The point, however, is that simian sexual behaviour is qualitatively different from that of other mammals: regardless of the terminology we use, mating during the ovarian cycle is commonly not tightly constrained either by hormones or by the likely time of ovulation. Further evidence is provided by Dixon's study of postpartum changes in hormones and sexual behaviour in common marmosets. Although sexual behaviour peaks around the first ovulation after a birth ("postpartum oestrus"), it is by no means confined to that time. Indirect recognition of this is shown both by Okayasu, who refers to 'oestrus' as "situation-dependent" in some groups of *Macaca fuscata*, and by Furuichi, who defines 'oestrus' in bonobos as "a period in which a female is sexually aroused, even if it is a nonfertile period". Nadler, in a

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REASONS

Kith and kin — kinship is an important influence on primate social life.

discussion of great apes, emphasizes the importance of female proceptive behaviour as an indicator of female sexual arousal and discusses evidence indicating that males may be largely responsible for mating outside the time of ovulation. The debate seems inconclusive, but this is no mere quibble about terminology. Many authors have drawn an artificial distinction between the occurrence of 'oestrus' in simians and the 'loss of oestrus' during human evolution, thereby obscuring a major change during the evolution of simians, rather than specifically during human evolution.

A discussion of conservation is now a standard feature of IPS congresses and the 1990 Kyoto symposium focused on tropical rainforests, with emphasis on the interaction of development and conservation. Sustainability is now in vogue, although it means different things to different people (J. Robinson). Studies are reported on traditional use of rainforest by hunter-gatherers (M. Ichikawa) and on shifting cultivation in South-East Asia (K. Tanaka), together with a discussion of 'ethnobiology' in relation to conservation

(D. Posey). Regrettably, there is a local flavour here, too: logging in South-East Asian insular countries is heavily influenced by a huge Japanese demand for timber. Japan allegedly imports 50 per cent of all-round timber harvested from the world's tropical forests. There is a particular problem with destruction of mangrove swamps in South-East Asia for wood-chip production (Y. Kuroda). Although this makes way for local farming of shrimps (also destined mainly for Japan), the overall cost to fisheries is greater than any commercial gain from felling mangrove forest, as is reported by T. Struhsaker in a survey of five case studies including one in Malaysia. There is, however, an encouraging note in that a new conservation policy of the Japanese government, aimed at promoting sustainability and recycling of paper, was presented to the IPS congress. A readiness for dialogue was also indicated. □

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fine for those familiar with the terminology, the description of this progress may not be totally comprehensible to the general reader. More explanation would have conveyed this exciting story to an even wider audience: for example, simple diagrams explaining concepts such as linkage, restriction fragment length polymorphism, DNA probes and recombination would have made what is an otherwise very well written book more accessible. Molecular genetics has shifted the approach to the aetiology of diseases not classically regarded as 'genetic'. The public must understand the dramatic nature of such advances if they are to support genome research. I hope that this book will contribute to the communication of such ideas.

The last part of the book deals with the steps leading to the first identification of a defined cause of Alzheimer's, mutations in the amyloid precursor protein (APP) gene, and subsequent advances. A second gene, on chromosome 14, has been located, and is linked to the disease in Hannah's family. A coverage of the importance of apolipoprotein E brings the story completely up to date.

The evolution of the term 'Alzheimer's disease' is documented throughout the book. Its extension in the 1970s to encompass what was previously known simply as senile dementia was crucial in attracting public attention and funding. Ironically, the research this success encouraged has now unequivocally demonstrated that Alzheimer's disease has many different causes. It remains to be seen if the causes are eventually all channelled through a common final pathway. If they are, then it may be easier to develop therapies that can be widely applied.

Pollen has done an excellent job in crediting the many workers in all the international research groups involved in the quest. A key lesson is the way in which linkage studies led to the rejection, at least for a while, of the APP gene as a candidate for the disease. One anecdote that does not appear in the book derives from the British practice of putting blue plaques on buildings where historical events occurred or notable people lived. St Mary's Hospital Medical School in London has one to mark where Fleming discovered penicillin, but for a while a plaque also appeared on the door of John Hardy's office. His research group had produced a replica reading: "this was where John Hardy demonstrated that APP was not the gene for Alzheimer's disease". The word 'not' had been crossed out. This book is crammed with human triumphs of both the intellect and the spirit, and deserves a wide readership. □

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Memory and molecules

John Collinge

Hannah's Heirs: The Quest for the Genetic Origins of Alzheimer's Disease. By Daniel A. Pollen. Oxford University Press: 1993. Pp. 296. \$25, £16.95.

A REVOLUTION is taking place in our understanding of degenerative diseases of the brain. By far the commonest of these is Alzheimer's disease: by the age of 75–80, 20 per cent of us may be affected by it. "The disease of the century", as Daniel Pollen refers to it, is in a sense born of the success of medicine in increasing the average lifespan of the population, and its treatment or prevention now stands as one of medicine's greatest challenges.

Only in the past two years have definitive causes of Alzheimer's disease been elucidated. Despite the sustained efforts of neuroscientists over several decades, the aetiology remained obscure: the nervous system is extremely complex and it is difficult to interpret whether a particular anatomical or chemical change is related to the underlying cause of the disease or, more likely, represents a sequel to the dramatic and devastating events of the neurodegenerative process.

A few cases of Alzheimer's were known to run in families, and their distribution suggested that an autosomal gene was responsible. Advances in molecular genetics provided a way of locating the gene and determining, albeit perhaps only in a few families initially, a defini-

tive cause of the disease. Researchers could then focus activities on a single protein and, from this foundation, it might be possible to unravel the complex mixture of genetic and environmental factors behind the commoner sporadic type of the disease.

In *Hannah's Heirs*, Pollen, a Boston neurologist with wide experience in this work, describes the search for Alzheimer's genes from the perspective of some of the families afflicted by this devastating illness. His starting point is the story of Hannah's family. In the late 1800s in Byelorussia, 40-year-old Hannah began to suffer from progressive memory loss. Years later, one of her descendants turned up at the author's clinic suffering from the same disease; many members of the family had been similarly afflicted over the intervening five generations. The historical and social perspective is compelling — a vivid reminder of the tragedy and horror of an illness that afflicts the very characteristics that define us as human beings. The simple and unending courage of family members should be a source of motivation to all those involved in Alzheimer's research.

Pollen provides a background to the latest discoveries by outlining the fundamental contributions of Mendel, Alzheimer, Morgan, Crick and Watson before comprehensively detailing the remarkable recent advances in our understanding of the human genome. Although

Atoms in carbon cages: the structure and properties of endohedral fullerenes

D. S. Bethune, R. D. Johnson, J. R. Salem, M. S. de Vries & C. S. Yannoni

Encapsulating atoms or molecules inside fullerene cages could give rise to a myriad of novel molecules and materials. The existence of such species is now strongly supported by a growing body of experimental evidence. Fullerene-metal complexes generally thought to be endohedral are being produced and purified in milligram quantities, and their structure and properties are beginning to be explored.

THE notion that atoms could be trapped in fullerene cages¹ was soon supported by mass spectral evidence that lanthanum-fullerene complexes were produced by laser vaporization of lanthanum-impregnated graphite (see Fig. 1)². Some early studies disputed this idea^{3,4}, but subsequent experiments have provided further support. Until mid-1991 endohedral fullerenes were produced only in minute quantities and few experiments were reported. This situation changed dramatically in 1991 when techniques to produce metallofullerenes in bulk (by laser-⁵ or arc-vaporization^{5,6} of graphite-metal composites in helium) were developed, allowing the first detailed spectroscopic characterization of a metallofullerene⁶ and leading to numerous experimental studies of endohedral fullerenes. Theoretical methods to calculate the structure of these complex entities are also being developed.

What properties might endohedral fullerenes be expected to have? By analogy with alkali-doped fullerenes, in which electrons are donated to the fullerene cage by interstitial metal atoms⁷, the conductivity of solid metallofullerenes will depend on the internal dopant; some may be superconductors⁸. Caged lanthanide ions will have optical transitions involving unfilled *f*-shell orbitals which would be determined by the crystal field due to surrounding carbon atoms. The resulting energy levels could provide a basis for a metallofullerene laser. Encapsulation of refractory metals such as uranium may allow such species to be conveniently put into solution, or volatilized without resorting to fluorination. It has been proposed that endohedral fullerenes containing small polar molecules could be assembled into useful ferroelectric materials^{9,10}. Experimental progress has been hindered by difficulties in separating the endohedral complexes from other fullerene species: however, samples of pure metallofullerenes have recently been obtained and these suggestions may soon be put to the test. The production of fullerenes containing internal species complements the extensive recent work on fullerene chemistry involving reagents outside the carbon cage¹¹.

Here we review existing methods for producing metallofullerenes and describe the work that has been done to characterize their structure and physical properties. The latter has relied to a large extent on techniques that probe electronic structure, and has focused on the nature of charge transfer between the metal and the fullerene cage. We then discuss theoretical studies of these compounds and how these compare with experiment, and finally we consider the future prospects for detailed structural characterization and possible applications.

Production techniques

Metallofullerenes can be produced by simply adding metal to carbon vapour in a standard fullerene generator¹². Chai *et al.* produced the first macroscopic amounts of metallofullerenes by laser vaporization of a graphite-metal composite rod in a helium-filled tube-oven at high temperature⁵. Mass spectra of material sublimed from the recovered soot showed normal fuller-

enes, and peaks corresponding to La@C_{2n} with 2n = 60, 70, 74 and 82; the '@' notation denotes an endohedral complex⁵. (Despite the present lack of direct structural evidence, we will use the '@' sign to designate species that we presume have endohedral structures.) Only La@C₈₂ was detected by mass spectrometry in toluene solutions of the sublimate. Subsequently the laser vaporization technique was used to produce Y@C_{2n} and Y₂@C₈₂ species¹³, a large number of U_m@C_{2n} species including U@C₂₈ and U₂@C₆₀ (ref. 14), and a M@C₆₀ species, Ca@C₆₀ (refs 15, 16).

Most recent metallofullerene production has used the arc or resistive heating methods. Chai *et al.* reported production of detectable amounts of La@C₈₂ by arc-burning carbon electrodes packed with La₂O₃, graphite powder and pitch⁵. Following this lead, our group at IBM arc-vaporized a rod made by pressing and baking a graphite-La₂O₃-dextrin mixture⁶. We subsequently simplified this technique by using cored carbon electrodes packed with dry mixtures of metal (or metal-oxide) and graphite powders¹⁷⁻¹⁹. The arc-vaporization technique produces soots that contain numerous metallofullerenes. A relatively small subset of these are sufficiently stable and soluble to allow extraction from the soot with solvents such as toluene, pyridine and carbon disulphide (CS₂). This subset includes M@C₈₂ species (with M = Sc, Y and La^{6,18-28}), di-metal species including La₂@C_{80,82} (refs 20, 22) and Y₂@C_{80,82} (refs 18, 19, 22, 25), di- and tri-scandium metallofullerenes^{17-19,24,29}, and many other mono- and di-metal rare-earth metallofullerenes^{19,30}. A recently introduced method for producing fullerenes is plasma-torch vaporization of carbon black and, for metallofullerenes, metal oxide³¹. Yields of Y@C₈₂, Y₂@C_{82,84} and normal fullerenes comparable to those obtained with arc techniques have been achieved³¹. Another unusual method—operating a carbon arc in a He-Fe(CO)₅ atmosphere—reportedly produced bulk quantities of a FeC₆₀ complex^{32,33}. Recently a CoC₆₀ complex was detected in soot, but could not be extracted³⁴.

Production of metallofullerenes has generally been inefficient. Typically they constitute less than a few weight per cent of the extractable species (or $\leq 10^{-3}$ of the soot). Yields are limited in part by the reactivity and scant solubility of metallofullerenes. They have solubilities similar to higher fullerenes, dissolving better in pyridine and CS₂ than in toluene^{22,25}. Production efficiency improves if metal carbides are used as metal sources, or if the carbide-rich cathode deposit produced *in situ* during arc-vaporization of a metal-containing bar is 'back-burned' by reversing the arc polarity²⁶. The fraction of metallofullerenes (especially multiple-metal fullerenes) increases with increasing metal loading, although overall fullerene yield falls³⁰. Anaerobic preparation increases the yield of some metallofullerene species^{23,27,29}.

An increasing variety of species have been successfully encapsulated in fullerenes. Significant quantities of fullerenes containing rare-gas atoms have been produced and detected³⁵, and

results suggesting that CO (ref. 36) and CN (ref. 37) have been trapped in fullerenes have been reported. Large metallofullerenes, containing up to 3 or 4 lanthanum atoms in cages made from >100 carbon atoms, have been found^{38,39}. Much larger fullerene-related structures, including concentric multi-walled graphitic tubules⁴⁰⁻⁴³ and polyhedra^{43,44}, and single-walled nanotubes^{34,45,46}, have been made recently. Partial filling of the multi-walled tubules⁴⁷⁻⁴⁹ and polyhedra⁵⁰⁻⁵³ with metal carbides or metals has been reported. Recently, electron beams (in a transmission electron microscope) were used to pierce holes in these gigantic endohedral fullerenes⁵³ and to drive an initially encapsulated gold nanocrystal out through the wall of a graphitic polyhedron⁵². Filled tubules could conceivably serve as nanometre-scale wires, whereas encapsulated single-domain ferromagnetic nanocrystals might have applications in data storage or in ferrofluids. Production techniques essentially identical to those used for metallofullerenes have now created mixed-atom cages dubbed metallocarbohedrenes⁵⁴⁻⁵⁹.

Characterization

Characterization of samples containing the presumed endohedral complexes has the aim of finding which species are produced, assessing their relative abundance, stability, and chemical reactivity, and determining their structure. Numerous techniques have been brought to bear.

Mass spectrometry. Molecular-beam techniques and mass spectrometry have provided very powerful tools for fullerene

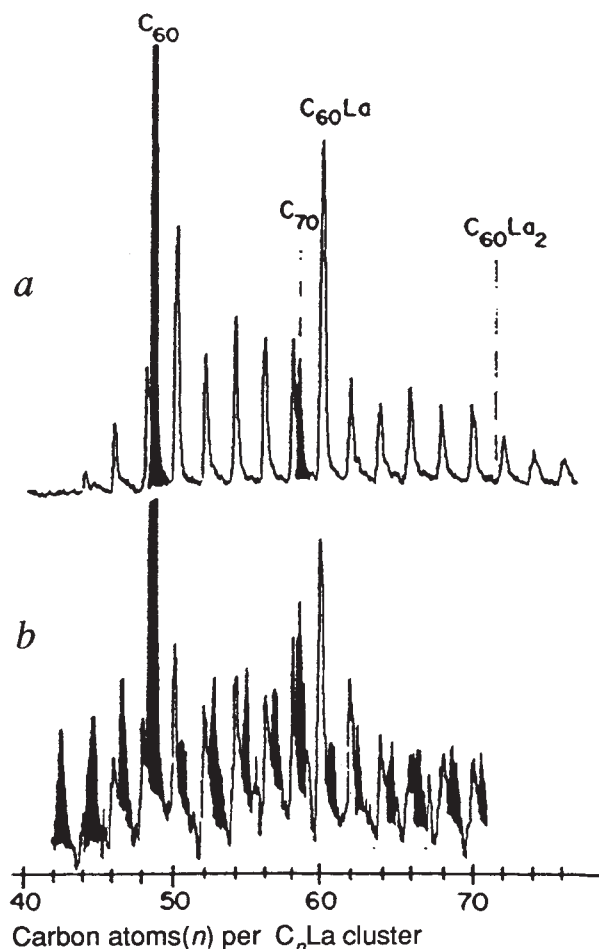


FIG. 1 Mass spectrum of $\text{La}@\text{C}_{2n}$ complexes and empty C_{2n} molecules (darkened peaks) produced by laser vaporization of a lanthanum-impregnated graphite disk. ArF ionization laser fluence in (a) was $1\text{--}2\text{ mJ cm}^{-2}$, and in (b) was $<0.01\text{ mJ cm}^{-2}$. (From ref. 2, with permission.)

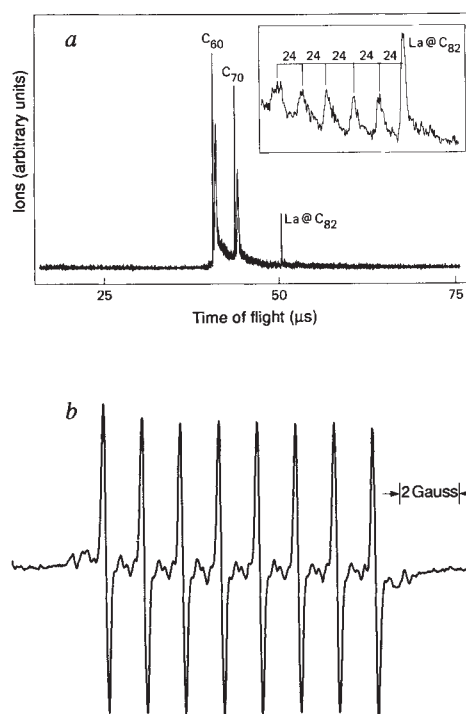


FIG. 2 a, Mass spectrum of the toluene extract of soot produced by arc-burning a La_2O_3 -graphite composite bar. Laser-desorption/ionization (wavelengths 248 nm and 193 nm respectively) time-of-flight mass spectrometry was used. The inset shows the fragmentation behaviour at high incident powers of ionization-laser radiation. b, EPR spectrum (9.5112 GHz) at ambient temperature of a degassed toluene solution of the same extract in 1,1,2,2-tetrachloroethane. (From ref. 6, with permission.)

research^{60,61}. This combination of techniques provided the crucial evidence^{1,62} that led to the discovery of fullerenes and endohedral fullerenes². The mass spectrum of our first lanthanum-fullerene sample is shown in Fig. 2a (ref. 6). The La-fullerene soot was inadvertently spiked with ^{13}C -enriched normal fullerene soot before toluene extraction. The spectrum is dominated by three mass peaks corresponding to C_{60} , C_{70} and LaC_{82} . The absence of ^{13}C -enriched satellites to the LaC_{82} peak confirms that this metallofullerene was not generated in the laser-desorption or ionization steps in the mass spectrometer but was present in the extract.

The inset in Fig. 2a shows that increasing the power of the incident (ionization) laser radiation causes the $\text{La}@\text{C}_{82}$ to fragment by shedding C_2 units, rather than by losing the metal atom, as originally reported by Weiss *et al.*⁶³. That observation, and the abrupt termination of the sequence of daughter-metallofullerene fragments at $\text{La}@\text{C}_{44}$, gave rise to the 'shrink-wrap' notion: the cage can lose carbon dimers and shrink about a trapped atom until, at a minimum size dependent on the trapped species, it bursts⁶³. An additional demonstration of how tightly the metals in metallofullerenes are bound was provided by Yeretizian *et al.*, who collided $\text{La}_2@\text{C}_{80}$ ions with a surface using a modified time-of-flight apparatus. No metal dissociation occurred even at 200-eV incident energy⁶⁴. Inert-gas/fullerene complexes made by gas-phase ion-molecule reactions, when subjected to secondary gas-phase collisions, show a similar reluctance to part even with helium⁶⁵⁻⁷⁴. Here again C_2 units are lost preferentially, clearly implying that the inert gas atom must be inside the cage.

Ion cyclotron resonance mass spectrometry was used by Weiss *et al.* to show that the metals in metallofullerene ions are shielded from chemical attack⁶³. Recently, gas-phase association

reactions in Fourier-transform-ICR machines have been used to create and trap exohedral metal–fullerene complexes^{75–78}. This technique allowed McElvany to compare directly the reactivity of N_2O with exohedral YC_{60} to its reactivity with YC_{60} complexes formed by a laser vaporization method. The exohedral complex reacted readily to give YO, whereas the directly produced complex showed essentially no reaction, despite exposure to a higher ($\times 10^3$) concentration of N_2O (ref. 78). This experiment confirms the early results of Weiss *et al.*⁶³ and provides compelling additional evidence for the endohedral nature of the laser-produced complex.

The properties of metallofullerenes exacerbate some difficulties in using mass spectrometry for fullerene characterization. It is found that the mass spectrum obtained depends drastically on how the sample molecules are put into the gas phase^{19,79}. Metallofullerene soot, analysed by laser-desorption mass spectrometry, contains a wide variety of metallofullerenes; their relative abundances are similar to the corresponding bare fullerenes ($M@C_{60}$ is typically the most prominent). But only a few metallofullerenes survive vacuum sublimation, notably $M@C_{82}$, and to some extent, $M@C_{74}$ and $M@C_{80}$ (ref. 19). A recent study⁷⁹ comparing laser- and thermal-desorption mass spectra of metallofullerene raw soots concluded that trivalent elements preferentially form endohedral complexes involving 60, 74 and 82 carbons, and that these species can survive slow thermal desorption. In contrast, divalent metals, such as Ca, Sr, Sm, Eu and Yb, preferentially form $M@C_{60}$ species that do not survive thermal desorption.

A difficulty that arises if photoionization is used is that the ionization cross-sections may vary, so ion peak heights may not indicate relative abundances correctly. This effect is notably exaggerated when normal and metallofullerenes are compared using 193-nm laser ionization, because ionization of the former requires two such photons, whereas metallofullerenes sometimes require only one, and are therefore detected with much higher efficiency^{4,19}.

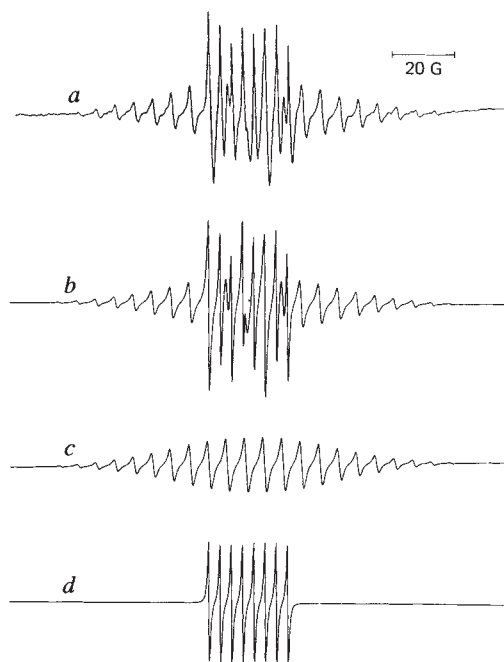


FIG. 3 The EPR spectrum of a toluene solution of toluene extract from soot produced by arc-vaporization of a carbon electrode with a scandium-packed core. (a) Experimental spectrum, (b) superposition of two simulated spectra arising from an unpaired electron spin coupled to (c) three equivalent scandium nuclei and (d) a single scandium nucleus. (From ref. 17, with permission.)

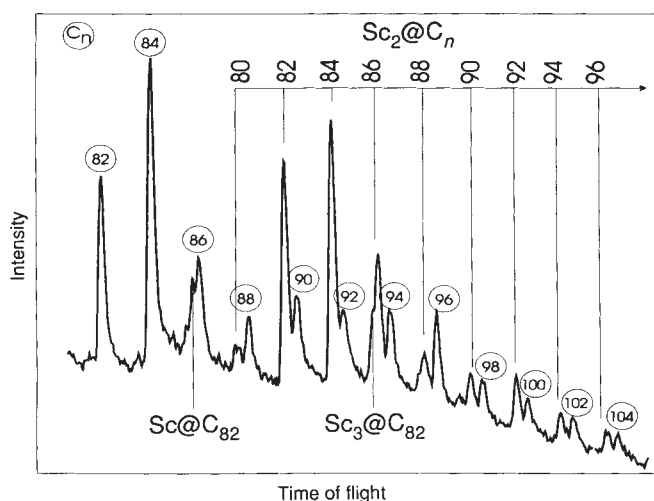


FIG. 4 Mass spectrum of the scandium metallofullerene sample. The dominance of $Sc_2@C_{82,84,86}$ species is evident.

Electron paramagnetic resonance. EPR spectroscopy has yielded detailed information about the electronic structure and chemical nature of several metallofullerenes, even though they constituted at most 2% (and often much less) of the fullerene extracts. The first metallofullerene EPR spectrum, EPR of $La@C_{82}$ in solution, is shown in Fig. 2b (ref. 6). The observation of eight equally-spaced lines provides dramatic and unequivocal confirmation of isotropic electron–nuclear hyperfine coupling (h.f.c.) to ^{139}La with nuclear spin quantum number 7/2 (ref. 80); the electron *g*-value of 2.0010, close to that measured for the C_{60} radical anion^{81–84}, indicates that a single unpaired electron (doublet state) resides in the lowest unoccupied molecular orbital (LUMO) of the fullerene cage. Each ^{139}La hyperfine line shows ^{13}C hyperfine satellites, proving that the unpaired electron couples to both the ^{139}La and the fullerene-cage carbon atoms. The 1.2-G ^{139}La h.f.c. is very small compared to 50-G h.f.c. measured for La^{2+} substituted in CaF_2 (ref. 80) and –196-G h.f.c. calculated for La^{2+} using unrestricted Hartree-Fock (UHF) wavefunctions. Therefore, it was concluded⁶ that the lanthanum atom must be in its preferred 3+ oxidation state, and that the structure is most appropriately written $La^{3+}C_{82}^{3-}$. Subsequent theoretical calculations^{85,86} and EPR studies^{23,28} have strongly supported this conclusion. Recent results on purified $La@C_{82}$ (ref. 28) confirm that the EPR spectra were correctly assigned.

EPR spectra obtained for $Y@C_{82}$ showed that the yttrium in this species is also in the 3+ oxidation state^{13,18,25}. A $Y_2@C_{82}$ species observed by mass spectrometry showed no EPR spectrum, suggesting that it is diamagnetic. Partial isolation of $Y@C_{82}$ has been achieved using high-performance liquid chromatography (HPLC) with on-line EPR detection to give an EPR-active fraction that contained C_{84} with a trace of $Y@C_{82}$ (ref. 87).

The EPR spectrum of an extract containing scandium metallofullerenes (Fig. 3a) shows considerably more structure than lanthanum or yttrium spectra^{17,24}. This spectrum was modelled (Fig. 3b) by superimposing two spectra, one of which simulates h.f.c. to a single ^{45}Sc nucleus with spin 7/2 (Fig. 3d), and the other simulates h.f.c. to three equivalent ^{45}Sc nuclei (Fig. 3c). Comparison of the experimentally observed 3.82-G h.f.c. for the mono-scandium species with 60-G h.f.c. observed for Sc^{2+} in CaF_2 (ref. 88) and –120-G h.f.c. calculated for isolated Sc^{2+} (using UHF wavefunctions) suggests that the scandium in $Sc@C_{82}$ is also in the 3+ state. The ^{45}Sc h.f.c. for $Sc_3@C_{82}$ (6.8 G) is only slightly less than 8.5-G h.f.c. measured for

molecular Sc_3 in an argon matrix at 4.2 K (ref. 89). The extent of charge transfer to the cage is unknown in this case.

The mass spectrum of the scandium material, shown in Fig. 4, reveals that Sc@C_{82} and $\text{Sc}_3\text{@C}_{82}$ are actually minor components compared with the di-scandium species $\text{Sc}_2\text{@C}_{2n}$ ($2n = 80, 82, 84, 86, \dots$). The latter are EPR-silent, whereas Sc_2 isolated in a neon matrix at 4.2 K has an EPR-active quintet ground state^{90,91}. The difference in spin multiplicity between the matrix-isolated and fullerene-caged di-scandium species may indicate bonding between the fullerene cage and the trapped atoms.

Other EPR and mass spectrometric studies have shown that several different metallofullerene species with the same chemical formula are formed in a metal-graphite arc-burn^{18,21,23}. The nature of the difference between these species is still a mystery. The EPR technique has also been used to study the stability in air of the lanthanum and scandium metallofullerene isomers^{23,26,27}.

Photoelectron spectroscopy, Mössbauer spectroscopy and XAFS. Several other analytical methods have been applied to metallofullerenes. X-ray photoelectron spectroscopy (XPS) has been used to characterize solid fullerene-metallofullerene films containing La@C_{2n} , Y@C_{2n} and $\text{Y}_2\text{@C}_{2n}$. The XPS spectra indicate that the metal atoms are protected from chemical attack and have a formal valence of 3+ (ref. 13). Similarly, XPS spectra of a film containing U@C_{2n} were consistent with a formal uranium valence of 4+ (ref. 14). For Ca@C_{60} entrained in a supersonic helium beam, ultraviolet photoelectron spectroscopy (UPS) carried out by Wang *et al.*^{15,16} gave an electron affinity of 3 eV (0.3 eV higher than for C_{60}). The spectra also indicated that calcium donates both valence electrons to the C_{60} cage.

Mössbauer spectroscopy is extremely sensitive to valence state for certain atoms. Pradeep *et al.*³² applied this technique to their FeC_{60} sample and concluded the iron was near zero valent, whereas Su *et al.* found that the europium in a soot containing many $\text{Eu}_m\text{@C}_{2n}$ species was trivalent⁹².

One of the few structural probes that can be applied to dilute samples of material is extended X-ray absorption fine structure (EXAFS). One EXAFS study of yttrium-metallofullerenes concluded that the Y atom is outside the cage⁹³. On the other hand, a more recent study on material containing YC_{82} and Y_2C_{82} (at 10 K and ambient temperature), indicated that the Y atoms are endohedral⁹⁴. In the latter study the data were modelled assuming 2 closest shells of 6 carbon atoms each, at 2.4 and 2.9 Å. These results are consistent with recent theoretical studies of a presumably similar species, La@C_{82} , which find the optimum metal-ion position to be inside the cage and ~2.5 Å from the nearest carbon atoms^{85,86}.

Theory

One would like to understand theoretically the metallofullerene formation process, their favoured cage sizes and isomers, electronic structure, chemical behaviour and solid-state properties. This is a very tall order, but a good start has been made. Early electronic structure calculations for metallofullerenes considered a rigid C_{60} cage containing a central metal atom. Rosen *et al.*^{95,96} predicted that, for La@C_{60} , the C_{60} levels shift down uniformly and the three metal valence electrons transfer to the cage LUMO.

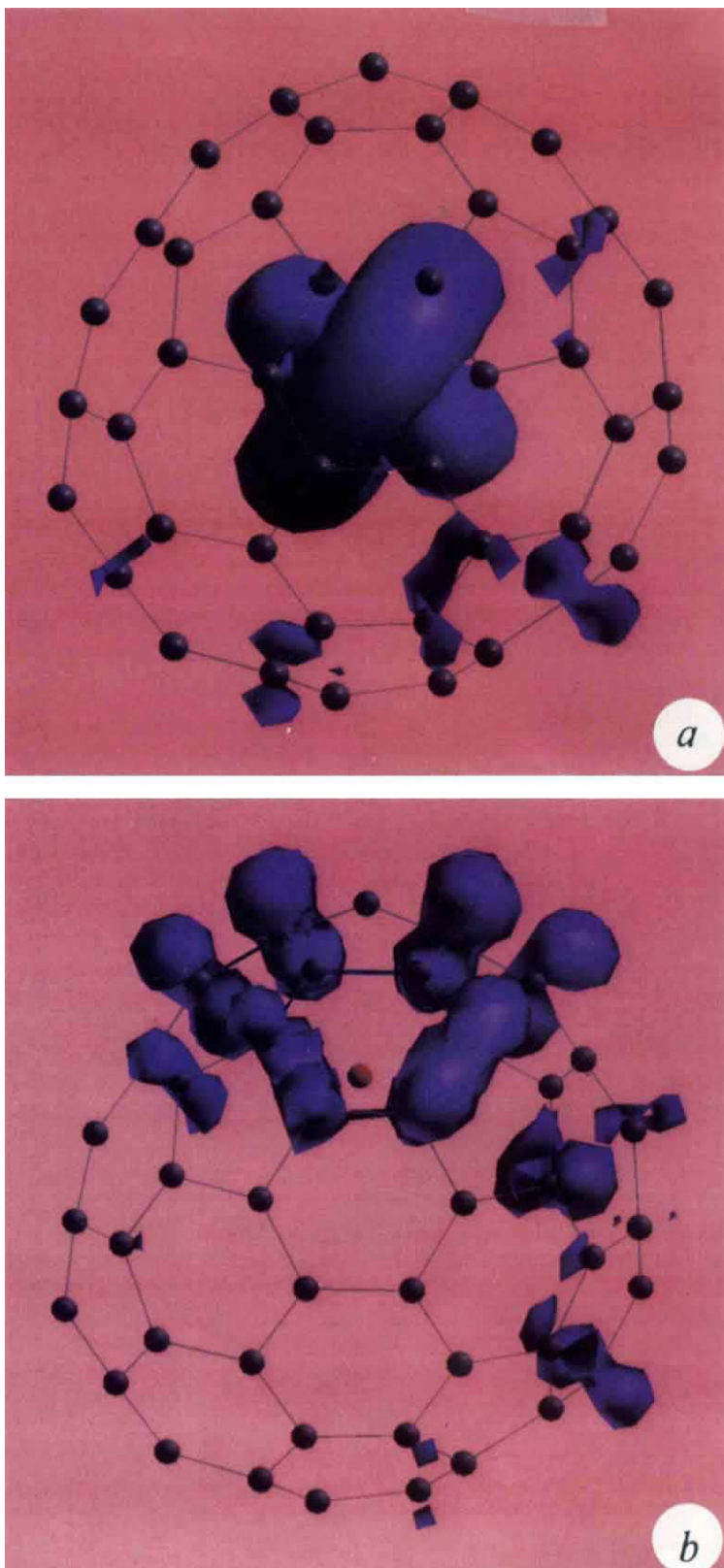


FIG. 5 Surface of constant electron density (0.01 electrons per unit of atomic volume (0.529 Å^3)) corresponding to the HOMO state of La@C_{82} with the lanthanum (red) located at the cage centre in the 2+ oxidation state (a), and at an energetically favourable asymmetric position in the 3+ state (b). The C_{82} cage (initially C_{3v} symmetry, but relaxed) is viewed perpendicular to a mirror plane. (From ref. 85, with permission.)

In contrast, a later study of this complex found a quartet ground state with only two of the lanthanum electrons transferred to the LUMO⁹⁷. Calculations with a central alkali-metal atom predicted that the metal loses its valence electron to the cage⁹⁸. Two calculations on Ca@C_{60} predicted that both metal valence electrons are transferred to the cage^{99,100}, whereas a third calculation predicted a triplet ground-state complex involving monovalent calcium⁹⁷. The minimal perturbation and uniform shift of the cage molecular orbital energy levels typically found in these calculations result from the central location of the metal atom, which minimizes overlap between metal and cage orbitals and (if Jahn-Teller distortion is neglected) preserves the high cage symmetry.

Whereas endohedral rare gas atoms may be centred in C_{60} , metals may favour off-centre positions¹⁰¹⁻¹¹⁴. Motion of endohedral atoms inside fullerene cages has been analysed by a number of groups^{103-108,115,116}. Theoretical calculations are complicated by the loss of symmetry for off-centre metal positions, and by the lower-symmetry, larger cages typical of experimentally observed metallofullerenes. Nevertheless, Laasonen *et al.* recently carried out *ab initio* calculations for La@C_{82} (relaxed C_{3v} cage)⁸⁵. Their results showed that the lanthanum is in a 2+ oxidation state if it is located externally, centrally, or on the 3-fold axis (see Fig. 5a). But for lower-symmetry (C_s) sites the third valence electron is lost to the cage; the resulting highest occupied molecular orbital (HOMO) is illustrated in Fig. 5b. The energy calculated for this off-centre configuration is 3.5 eV lower than for the configuration with a centrally located La. A subsequent semi-empirical calculation by Nagase *et al.* led to similar conclusions⁸⁶. Andreoni *et al.* have also carried out *ab initio* calculations for M@C_{60} ($\text{M} = \text{K}, \text{Na}, \text{Al}$ and La)^{109,110}. Other recent theoretical work investigated the structures of numerous M@C_{28} species¹¹⁷⁻¹²⁰, and the electronic structure of $\text{Sc}_3\text{@C}_{82}$ (ref. 121).

Why do only certain cages seem favourable for stable metallofullerene formation? In general, it is difficult to understand how fullerenes form. Some guidelines such as the 'isolated pentagon rule'^{122,123} seem to hold. Hückel molecular-orbital theory has been used by Manolopoulos and Fowler to estimate the HOMO-LUMO gaps of isolated-pentagon fullerene isomers. For normal fullerenes this approach identified successfully the preferred C_{60} , C_{70} and C_{76} cages, but was less successful for higher fullerenes¹²⁴⁻¹²⁸. By studying fullerene-dianions they identified C_{74}^{2-} , C_{82}^{2-} and C_{88}^{2-} as promising closed-shell candidates for metallofullerene formation^{129,130}. But the EPR results for La@C_{82} (ref. 6) demonstrate clearly that simple 'shell closing' models^{5,129} are inadequate. (This is unfortunate because *a priori* methods are too costly for survey work.) One imaginative model for the formation of La@C_{82} is based on reacting an *exohedral* LaC_{60} with carbon to give an endohedral complex with the metal residing in a 22-atom side cage attached to the C_{60} unit (ref. 131).

From an empirical standpoint, it is intriguing to note that in an early paper on formation of fullerenes in flames¹³², mass spectra of the negative ions showed prominent peaks for C_{2n}^- with $2n = 50, 74, 80, 82$ and 84 . All of these (except C_{50}) form stable, soluble metallofullerenes, suggesting that the electron affinity of empty fullerenes is related to metallofullerene stability, and even that the same isomers occur for both the hollow and metal-containing species. Thus an attempt to guess the cage isomer for La@C_{82} (ref. 86) from NMR data on C_{82} (ref. 133) may be reasonable.

Status, directions and prospects

The idea that atoms can be trapped in fullerenes to form stable metallofullerenes has been consistently supported by spectroscopic, fragmentation and chemical evidence. The reactivity of deliberately synthesized exohedral metal-fullerene complexes compared with the relative inertness of arc-produced metallofullerenes provides particularly convincing, albeit indirect evidence

that the latter are indeed endohedral^{163,78}. An elegant experiment by Saunders *et al.*³⁵ showed the existence of minute amounts of He@C_{2n} species in fullerene soot prepared using standard methods¹². Using ultra-sensitive mass spectrometry they found that at temperatures of $\sim 800^\circ\text{C}$, the fullerenes released ^4He and ^3He in the ratio expected for tank helium rather than atmospheric helium, proving that the gas was trapped in the fullerenes during formation in the presence of the tank helium. Similar results were obtained with neon³⁵. The unprecedented observation of stable inert-gas-carbon complexes can only be explained if the inert-gas atoms are encapsulated. Thus, despite the lack of direct structural data, the accumulated evidence leaves little doubt that endohedral fullerenes exist.

Application of most traditional spectroscopic techniques and structural techniques (X-ray, electron or neutron diffraction) to endohedral fullerenes has been impeded by the difficulty in producing pure samples. The technique of EPR spectroscopy, which selectively detects radical metallofullerenes, has provided the most detailed electronic structural information to date, particularly the oxidation state of the metal atom and the spin multiplicity of the complexes. Important progress toward purification of metallofullerenes has recently been reported by two groups using multiple HPLC stages to obtain pure samples of $\text{Sc}_2\text{@C}_{2n}$, with $2n = 74, 82$ and 84 (refs 27, 29), and pure La@C_{82} (see Fig. 5)²⁸. As a result of these purification efforts the first ultraviolet, visible^{28,29} and infrared²⁸ spectra of these materials have been obtained, and scanning tunnelling microscopy (STM) images of metallofullerenes on a surface have been made¹³⁴. The STM images, taken of Sc@C_{74} and $\text{Sc}_2\text{@C}_{74}$ on a silicon surface, show the molecules to be spherical with apparent cage diameters

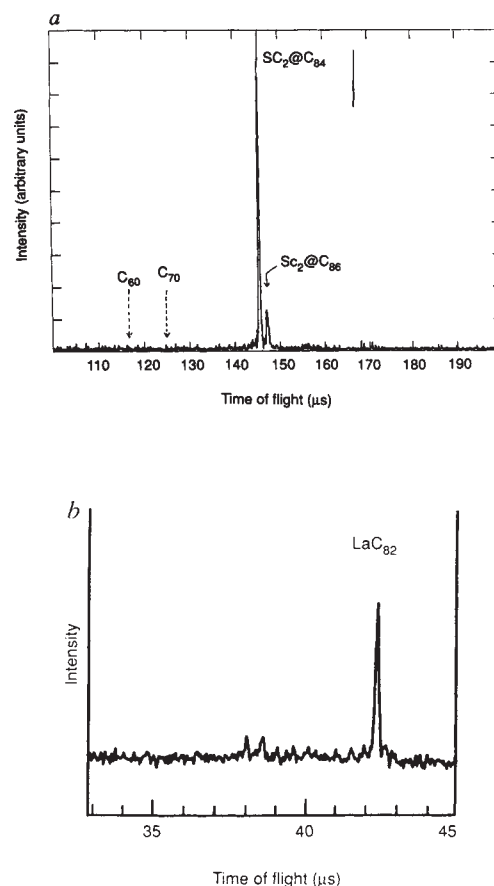


FIG. 6 Mass spectra of the first purified metallofullerene species. a, $\text{Sc}_2\text{@C}_{84}$ (from ref. 29, with permission); b, La@C_{82} (from ref. 28, with permission).

of ~ 9.5 Å. Exploration of the properties of these novel materials should accelerate in light of these developments.

Important questions remain as spurs for further research. Can alternative routes to bulk production of endohedral species be developed, perhaps using cage-opening reactions or direct rational synthesis? Which species can be encapsulated? Can large quantities of purified endohedral fullerenes be efficiently made?

Above all, what are their various physical properties? Beyond their scientific interest, the possibility of developing important applications for endohedral fullerenes will hinge on the answers to such questions. □

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The protein tyrosine kinase JAK1 complements defects in interferon- α/β and - γ signal transduction

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We have produced a cell line which lacks the protein tyrosine kinase JAK1 and is completely defective in interferon response. Complementation of this mutant with JAK1 restored the response, establishing the requirement for JAK1 in both the interferon- α/β and - γ signal transduction pathways. The reciprocal interdependence between JAK1 and Tyk2 activities in the interferon- α pathway, and between JAK1 and JAK2 in the interferon- γ pathway, may reflect a requirement for these kinases in the correct assembly of interferon receptor complexes.

THE interferons (IFNs) can induce an antiviral state and modulate cell growth, differentiation and the immune response. IFN- α/β (type I) and IFN- γ (type II) induce distinct but overlapping sets of genes through independent cell surface receptors (reviewed in refs 1, 2). Binding of either type of IFN to a receptor rapidly induces tyrosine phosphorylation of latent transcription factors (reviewed in ref. 3). IFN- α/β treatment results in phosphorylation of the 113K (p113), 91K (p91) and 84K (p84) components of the α -subunit of interferon-stimulated gene factor 3 (ISGF3)⁴⁻⁷. The activated α -subunit combines with the 48K (p48) DNA-binding γ -subunit^{8,9} and, on translocation to the nucleus, ISGF3 binds to *cis*-acting DNA response elements termed IFN-stimulable response elements (ISREs) to initiate transcription¹⁰⁻¹². The p91 and p84 components of ISGF3 result from alternative splicing and differ only in a 38 amino-acid extension at the carboxy terminus of p91 (ref. 6). These polypeptides are also phosphorylated on tyrosine in response to IFN- γ ¹³. Consistent with a central role for p91 in both response pathways, characterization of mutant U3A has shown p91 to be absolutely required for the IFN- α and - γ responses of a wide spectrum of genes¹⁴. The γ activation sequence (GAS) was first identified in the GBP gene^{15,16}. Recently, p91 has been shown to bind to the GBP GAS¹³ and to a GAS consensus sequence motif in a number of additional genes. For some of these, additional DNA elements and factors may also be required to mediate the primary IFN- γ response¹⁷⁻²⁰.

The lack of recognizable protein tyrosine kinase domains in the cloned IFN- α and - γ binding subunits of the IFN receptors^{21,22} implies that additional receptor or non-receptor proteins must provide the tyrosine kinase function(s) in these pathways. Furthermore, a subunit of the IFN- α receptor has been shown to be phosphorylated on tyrosine on ligand

binding²³. Mutational studies of the IFN- γ receptor have defined two cytoplasmic domains necessary for biological function, a membrane-proximal region and a C-terminal sequence including an essential tyrosine^{24,25}.

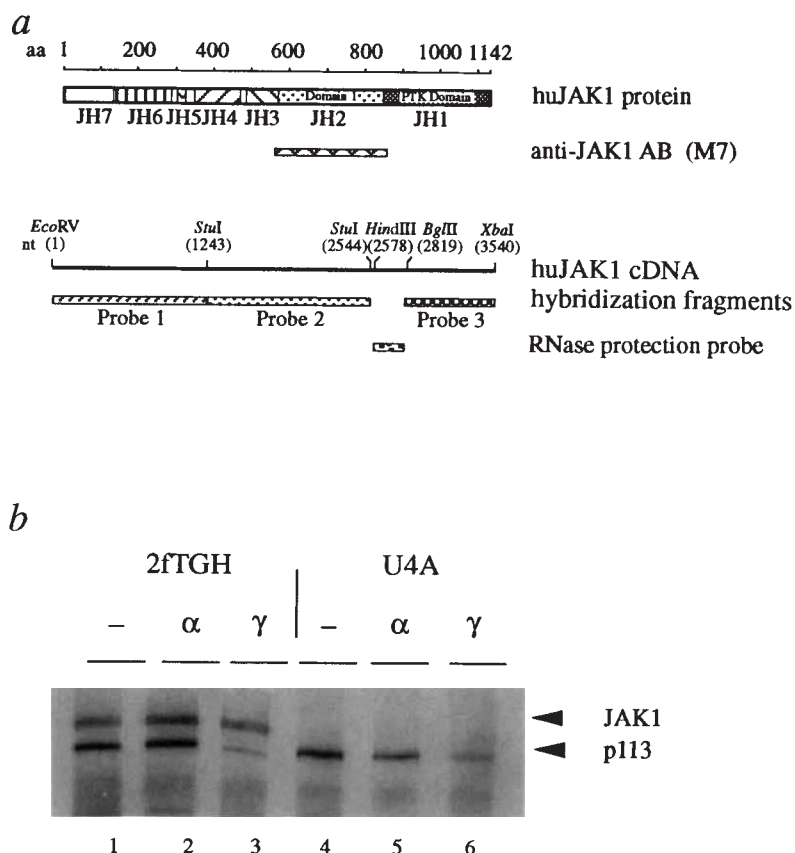
The complementation of IFN-resistant mutant cell lines has provided direct evidence for the involvement of the JAK family of non-receptor protein tyrosine kinases in the IFN response pathways. This family has three known members, JAK1 (refs 26-29), JAK2 (refs 26, 28, 30) and Tyk2 (ref. 31). Each is about 130K in mass and is characterized by the presence of a classical C-terminal protein tyrosine kinase domain, an adjacent kinase or kinase-related domain and five further domains of substantial amino-acid similarity with other members of the JAK family extending towards the amino terminus²⁸.

Two approaches, using drug-selectable and cell-surface markers under the control of IFN-inducible promoters, have been used in the mutant work. Using drug selection, Velazquez *et al.*³² first showed, by complementation of mutant U1A (initially coded 11,1; ref. 33), that Tyk2 is essential for the IFN- α response. Using the alternative cell surface marker approach, we show by complementation of mutant γ 1A a similar absolute requirement for JAK2 in the IFN- γ response (D. Watling *et al.*, accompanying paper⁵¹). Consistent with this, Tyk2 and JAK2 are phosphorylated on tyrosine in response to IFNs- α and - γ , respectively (D. Watling *et al.*, accompanying paper⁵¹; G.B., L. Velazquez, M. Scrobogna, M. Fellous and S.P., unpublished results; K. Shuai, A.Z., A.F.W., A.G.H., H. Sadowski, M. Z. Gilman and J. E. Darnell Jr, manuscript in preparation; O.S., J.N.J., J. Schlessinger and D. E. Levy, manuscript in preparation).

Here we present definitive evidence that the third known member of the JAK family, JAK1, plays an essential role in both the IFN- α and - γ response pathways. Mutant U4A, completely defective in response to IFNs- α and - γ ³⁴, lacks JAK1 and is

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FIG. 1 a, Diagram of the human JAK1 protein showing the open reading frame of 1,142 amino acids, the seven (JH1-7) JAK family homology domains and the region to which the antibody M7 used throughout was raised^{27,28}. A limited restriction map of the human JAK1 cDNA²⁷ and the probes used in the northern and Southern (Fig. 2) and RNase protection (Fig. 4) assays are shown below. b, Analysis of JAK1 protein in 2fTGH and mutant U4A cells with and without prior treatment with IFN. Whole-cell lysates were immunoprecipitated and analysed by SDS-PAGE and western transfer with antibodies to JAK1 and, as an internal control, p113. Treatment of cells with 500 IU ml⁻¹ of IFN- α (a mixture of highly purified human subspecies, Wellferron, 1.5×10^8 IU mg⁻¹ protein, provided by Wellcome Research Laboratories, Beckenham, UK) or recombinant IFN- γ (4×10^7 IU mg⁻¹ protein, a gift from G. Adolf, Ernst Boehringer Institut für Arzneimittelforschung, Vienna, Austria), as indicated, was for 15 min at 37 °C. Parental 2fTGH and mutant U4A cells, tissue culture conditions and selective media have been described previously^{14,33,34}. Whole-cell lysates from $5-7 \times 10^6$ cells were prepared in lysis buffer (0.5% NP-40 or Triton X-100, 10% glycerol, 50 mM Tris-Cl pH 8.0, 200 mM NaCl, 0.1 mM sodium ortho-vanadate, 50 mM NaF, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, 3 mg ml⁻¹ aprotinin, 1 mg ml⁻¹ leupeptin) as described⁷. JAK1 and p113 were immunoprecipitated from precleared cell extracts with antisera to JAK1²⁷ and to p113^{4,7} in the presence of protein A Sepharose (Pharmacia) as described previously^{6,13} and analysed by SDS-PAGE (7%) and western transfer. Detection was by enhanced chemiluminescence (ECL) (Amersham Life Science) and fluorography (Fuji RX). Peroxidase conjugated secondary antibodies were rabbit anti-mouse (DAKO) or goat anti-rabbit (Jackson ImmunoResearch).



complemented by either human or murine JAK1. Consistent with this, JAK1 has independently been shown to be phosphorylated on tyrosine in response to IFNs- α and - γ in a number of human and murine cell lines (K. Shuai *et al.*, manuscript in preparation; O.S. *et al.*, manuscript in preparation; see also Fig. 6). As expected JAK1 is required for the phosphorylation of the transcription factor p91 in response to IFNs- α or - γ . Less predictably, it is also required, directly or indirectly, for phosphorylation of the p113 subunit of ISGF3 in response to IFN- α and for the phosphorylation of Tyk2 or JAK2 in response to IFNs- α or - γ , respectively.

U4A cells lack the protein tyrosine kinase JAK1

The mutant cell line U4A was derived³⁴ from the human cell line 2fTGH in which expression of the drug-selectable *gpt* (guanine/hypoxanthine phosphoribosyl transferase) gene from *Escherichia coli* is controlled by an IFN- α /IFN- β -inducible promoter from the 6-16 gene³³, by mutagenesis and selection for resistance to IFN in medium containing IFN- α and 6-thioguanine. Here complementation of the mutation and restoration of the IFN response with JAK1 was by selection in medium containing HAT (hypoxanthine, aminopterin, thymidine) and IFN- α .

The domain structure of JAK1²⁸, the region to which the antiserum M7 (ref. 27) used throughout this study was raised, the JAK1 complementary DNA²⁷ and the cDNA fragments and RNase protection probes used in Figs 2 to 4 are shown diagrammatically in Fig. 1a. Extracts from IFN-treated and control 2fTGH and mutant U4A cells were analysed for JAK1 protein by sequential immune precipitation and western transfer with antisera to JAK1 and, as an internal control, the p113 component of ISGF3 (Fig. 1b). Extracts from U4A cells lack JAK1 but contain normal levels of p113 (lanes 4-6, Fig. 1b). Consistent with this, U4A cells express a truncated JAK1 messenger RNA of about 1.4 kilobases (kb) (Fig. 2a, lane 4). In contrast, 2fTGH cells express the expected mRNA of about 5.4 kb (Fig. 2a, lanes 1-3). JAK1 mRNA has been reported to be induced by IFN- γ

in human monocytes²⁹. Here no significant stimulation of JAK1 protein (Fig. 1, lanes 1-3) or mRNA (Fig. 2a, lanes 1-3) was observed in response to IFNs- α or - γ . Using the more quantitative RNase protection assay, a maximum two- to threefold stimulation of JAK1 mRNA in response to prolonged (17 h) treatment with 1,000 IU ml⁻¹ IFN- γ was, however, observed (M.M., C.L. and I.M.K., data not shown). The nature of the truncated JAK1 mRNA expressed by U4A cells was analysed further by sequential reprobing of the northern transfer used in Fig. 2a with the 5' (Fig. 2b), central (Fig. 2c) and 3' (Fig. 2d) cDNA probes described in Fig. 1a. Only the 3' probe detected the JAK1 mRNA fragment. In addition, analysis of U4A mRNA by RNase protection using the probe shown in Fig. 1a yielded a fragment of the expected size (Fig. 3c, lanes 1-3). Accordingly, the truncated mRNA must include nucleotides 2,578 to 2,819 of the published sequence²⁷ covered by the protection probe, plus sufficient sequence 3' of this to hybridize in a northern to the 3' cDNA fragment. The nature of the remainder of the truncated message is not clear. The longest available cDNA (referred to as 'full length'), and the cDNA fragment probes used (Fig. 1a), although covering the entire reading frame, do not contain significant sequence corresponding to the 3' and 5' untranslated regions of the message. The contribution of these 3' and 5' sequences to the truncated message is, therefore, not known. In addition, Southern analysis of genomic DNA digested with a variety of restriction enzymes and probed either with the 'full-length' cDNA for JAK1 or the cDNA fragment probes (Fig. 1a) failed to detect any difference between parental and mutant cells (Fig. 2e). Therefore, mutant cell line U4A is defective in JAK1 protein and expresses a 5' truncated JAK1 and mRNA without major rearrangement or deletion in the corresponding gene.

Complementation of mutant U4 with JAK1

Mutant U4A cells were stably transfected with constructs expressing either human or murine JAK1 and populations of

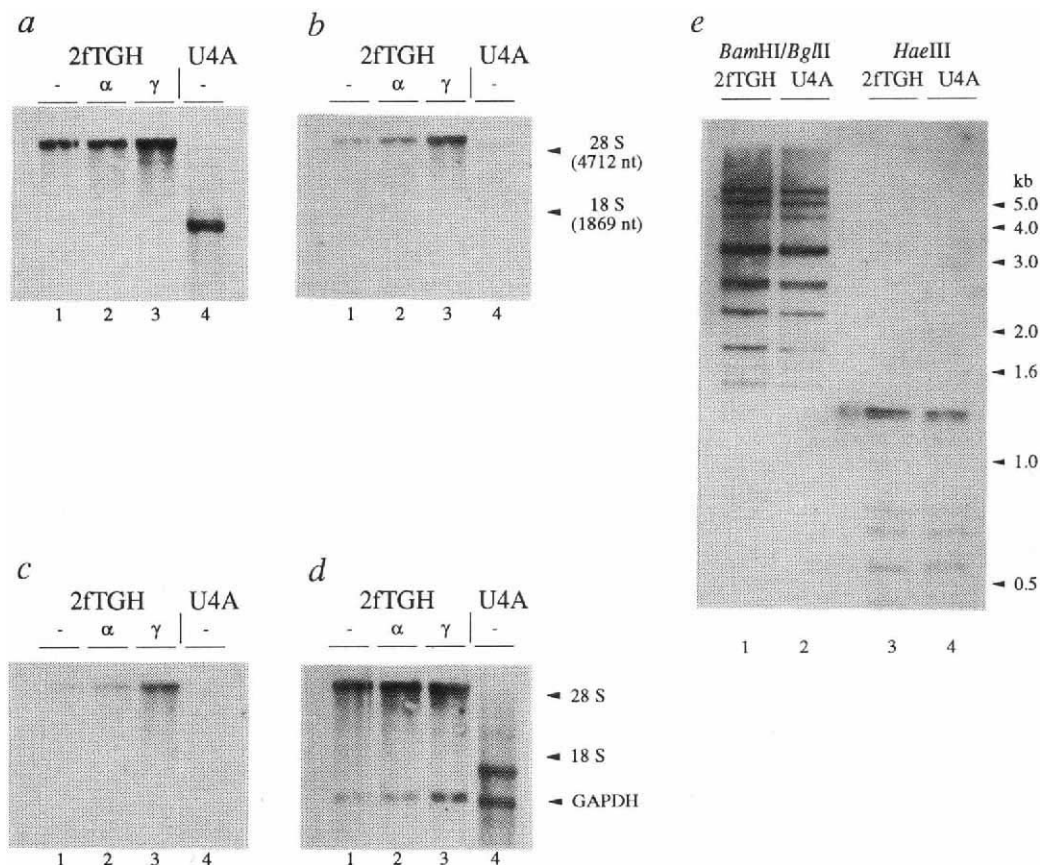
JAK1-expressing cells were isolated by selection for IFN- α -dependent growth in medium containing HAT. The JAK1 transfectants showed a growth pattern in selective medium identical to that of parental 2fTGH cells, that is, IFN- α or - β -dependent cell growth or death in medium containing HAT or 6-thioguanine, respectively. The mouse JAK1 protein and mRNA are considerably overexpressed in U4A/murine JAK1 transfectants (Fig. 3a, lane 3 and b, lane 2). The very low levels of endogenous JAK1 mRNA in parental 2fTGH cells was not detectable (lane 1) as total cytoplasmic RNA rather than poly(A)⁺ mRNA (as in Fig. 2) was used. Similar results were obtained when the mRNA and protein levels in U4A cells transfected with human JAK1 cDNA were analysed (Fig. 4c, lanes 7–9 and M.M. and I.M.K., data not shown). To date we have observed no difference between the human and the mouse JAK1 expression plasmids in the ability to restore the IFN-response of U4A cells. The U4A/murine JAK1 transfectants were used throughout, unless otherwise indicated.

Restoration of the transcriptional response

The induction of a variety of mRNAs in response to IFNs- α , - β or - γ in wild-type (2fTGH) and U4A cells and in U4A/JAK1 transfectants was assayed by RNase protection (Fig. 4a–c). Included are examples of mRNAs inducible only by type I IFNs (IFI-56K), only by type II IFN (INV, invariant chain of class II human leukocyte antigens (HLAs) and those inducible to varying degrees by either type of IFN (6-16, 9-27, IRF-1 and the p91, p84 and p48 components of ISGF3). The IFN- α and

- β response is primary for all genes tested. The IFN- γ response of IRF-1 is primary^{17,35,36}, whereas the DNA elements and factors mediating the primary and/or secondary responses of the remainder of the genes to IFN- γ have yet to be rigorously established. Mutant U4A is defective in the response of all genes tested to IFN- α and - γ ³⁴ (and, for example, lanes 1–3, Fig. 4a and b). The response to both types of IFN is completely restored in the U4A/murine JAK1 transfectants (compare lanes 4–6 with 7–9, Fig. 4a and lanes 4–9 with 10–15, Fig. 4b). In addition the kinetics of induction in response to IFN- α and - γ were indistinguishable in wild-type cells compared with the U4A/JAK1 transfectants (compare lanes 4–9 and 10–15, Fig. 4b). Similar results were obtained with the U4A/human JAK1 transfectants and with IFN- β with either type of transfectants. Examples are shown in Fig. 4c, where the response of U4A to IFNs- α and - β (lanes 1–3) is compared with that of wild-type 2fTGH cells (lanes 4–6) and U4A/human JAK1 transfectants (lanes 7–9). The response of U4A/murine JAK1 transfectants to IFN- β is included for comparison (lanes 10 and 11). The levels of human JAK1 and mRNA were also monitored in Fig. 4c and confirmed both the much higher levels of expression of this RNA in the U4A/human JAK1 transfectants (lanes 7–9) than in wild-type 2fTGH cells (lanes 4–6) and the presence of the JAK1 mRNA fragment discussed above (Fig. 2) in the U4A cells (lanes 1–3). In an additional series of experiments, expression of the class I HLAs in response to IFNs- α and - γ , and of class II HLAs in response to IFN- γ , was monitored by scanning with the fluorescence-activated cell sorter (FACS). Expression was completely

FIG. 2 Analysis of JAK1 mRNA and DNA in wild-type and mutant U4A cells. a–d, Northern analysis of poly(A)⁺ mRNA (7 μ g per lane) from control and IFN- α or - γ treated 2fTGH cells and from mutant U4A cells. The transfer was hybridized sequentially with a, the 'full-length' human JAK1 cDNA and b–d, the JAK1 cDNA 5', central and 3' fragment probes 1, 2 and 3 shown in Fig. 1a. After each hybridization the transfer was stripped and tested for any remaining probe by an overnight exposure. The positions to which 18S and 28S ribosomal RNAs migrated are indicated. In d, hybridization to GAPDH was included as a further loading control. Treatment with 500 IU ml⁻¹ of IFN- α or - γ was for 8 h or 18 h, respectively. e, Southern analysis of genomic DNA from 2fTGH and mutant U4A cells. DNA was digested with the indicated restriction enzymes and hybridized against the 'full-length' human JAK1 cDNA (Fig. 1a). The size markers to the right were from a commercial 1 kb ladder (BRL). Total cytoplasmic RNA isolation and northern transfer were as described¹⁴. Poly(A)⁺ mRNA was purified on oligo(dT)-cellulose spun columns (Pharmacia LKB). Southern analysis and hybridization conditions have been described previously¹⁴. The following fragments were isolated from the



human JAK1 cDNA²⁷ (Fig. 1a): 1.2 kb EcoRV-StuI (probe 1, nt 1 to 1,243), 1.3 kb StuI (probe 2, nt 1,243 to 2,578) and 0.7 kb BglII-XbaI (probe 3, nt 2,819 to 3,540). The hybridization probes were ³²P-dCTP-labelled to a specific activity of >10⁸ c.p.m. μ g⁻¹ using a multiprimer DNA labelling system (Amersham).

defective in U4A cells but normal in the U4A/JAK1 transfectants (J.B., D. Watling, M.M. and I.M.K., data not shown). It can be concluded that JAK1 is required for the primary response of all genes tested to both type I and type II IFNs.

Activation of primary transcription factors

Here, and in the subsequent sections, tyrosine phosphorylation was monitored by immune precipitation with specific antisera (against p91, p113, JAK1, JAK2 or Tyk2) followed by western analysis of the immune precipitates, first with antisera to phosphotyrosine (top panels Figs 5 and 6) and second, after stripping, with the p91, p113, JAK1, JAK2, or Tyk2, antisera as appropriate (bottom panels Figs 5 and 6). Phosphorylation on tyrosine of the p113, p91 and p84 components of ISGF3 occurs in response to IFN- α ⁷ and of p91 and p84 in response to IFN- γ ¹³. (Levels of p84 are usually lower than levels of p91 and phosphorylation of p84 is frequently difficult to detect^{7,13} as was the case here.) Phosphorylation of p91 and p113 in response to α and γ IFNs was, therefore, compared in wild-type, mutant and transfected cells. Whole-cell extracts were immunoprecipitated with antisera to p91 or p113 and the immunoprecipitates were subjected to electrophoresis and western analysis with antibodies to phosphotyrosine. Parental 2fTGH cells showed the expected pattern of phosphorylation of p91 in response to IFN- α or - γ and of p113 in response to IFN- α (Fig. 5a, lanes 1–3). No such phosphorylation was detected in mutant U4A (Fig. 5a, lanes 4–6).

Phosphorylation was completely restored in the U4A/JAK1

transfectants (Fig. 5a, lanes 7–9). Comparable levels of p91 and p113 were present in all of the extracts analysed (Fig. 5b). The more slowly migrating, tyrosine phosphorylated form of p91 is apparent in Fig. 5b (p91-P, lanes 2,3 and 8,9).

JAK1 is phosphorylated on tyrosine

Consistent with the requirement for JAK1 in both the IFN- α and - γ pathways, JAK1 phosphorylation was observed in wild-type 2fTGH cells in response to either type of IFN (Fig. 6Aa, lanes 2 and 3). In the absence of JAK1 (Fig. 6Ab, lanes 4–6) no phosphorylation was observed in mutant U4A cells (Fig. 6Aa, lanes 4–6). In the U4A/JAK1 transfectants, IFN-dependent phosphorylation was restored but against a constitutive level of phosphorylation (Fig. 6Aa, lanes 7–9). The site and basis for this constitutive phosphorylation is not known. It does not, however, result in detectable activation of the IFN response in these cells as, in the absence of added IFN, the transfectants neither grow in HAT medium nor express IFN-inducible genes (Fig. 4). Nor is constitutive phosphorylation of p91 or p113 observed in these cells (Fig. 5, lane 7). Similar constitutive phosphorylation is observed on overexpression of transfected Tyk2 (G.B., L. Velazquez, M. Scrobogna, M. Fellous and S.P., unpublished results) and JAK2 (D. Watling *et al.*, accompanying paper⁵¹).

Interdependence of JAK1, JAK2 and Tyk2

Tyrosine phosphorylation of Tyk2 and JAK2 in response to IFN- α or - γ , respectively, is dependent on the presence of JAK1. To define whether JAK1 is upstream or downstream of

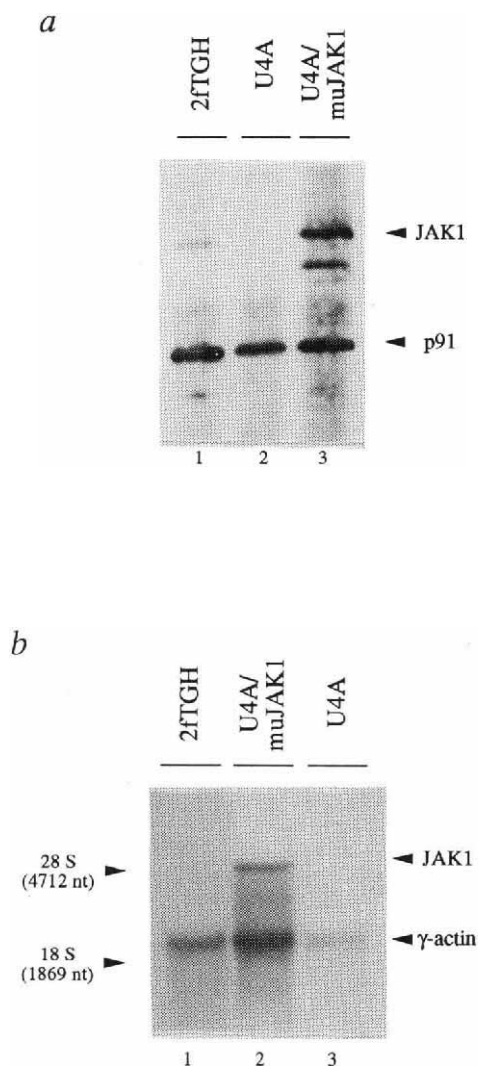


FIG. 3 Overexpression of JAK1 protein and mRNA in U4A/JAK1 transfectants. *a*, Whole-cell lysates from parental 2fTGH and mutant U4A and U4A/murine JAK1 transfectants were immunoprecipitated and analysed by SDS-PAGE and western transfer with antisera to human JAK1 and, as an internal control, p91^{6,7}. *b*, Northern analysis of total cytoplasmic RNA (10 μ g) from 2fTGH, mutant U4A and U4A cells transfected with murine JAK1. Hybridization was with the 'full-length' human JAK1 cDNA (Fig. 1a) with the γ -actin as a loading control. The positions to which the 18S and 28S rRNAs migrated are indicated. There is too little JAK1 mRNA in 2fTGH cells for it to be detected in preparations of cytoplasmic RNA (lane 1) compared with poly(A)⁺ mRNA (Fig. 2). Mutant U4A was complemented by transfection with a full-length murine JAK1 cDNA³⁰ downstream of the CMV promoter in pRK5 or human JAK1 cDNA²⁷ in the pCDM8 expression vector⁴⁹ in the presence of a neomycin-selectable marker plasmid. The G418-resistant population of stable transfectants were selected in medium containing HAT (20 μ g hypoxanthine, 0.2 μ g aminopterin and 20 μ g thymidine per ml) and IFN (500 IU ml⁻¹).

Tyk2 and JAK2 in the IFN signal transduction pathways, it was of interest to determine whether Tyk2 or JAK2 is phosphorylated in response to the appropriate IFN in the absence of JAK1. In wild-type 2fTGH cells, Tyk2 was phosphorylated in response to IFN- α (Fig. 6Ba, lane 2) but not IFN- γ (lane 3). No such phosphorylation was observed in mutant U4A (Fig. 6Ba, lane 5) but phosphorylation was restored in the U4A/JAK1 transfectants

(Fig. 6Ba, lane 8). The levels of Tyk2 protein were comparable in the extracts from each of these cell types and no change in these levels was observed in response to either IFN (Fig. 6Bb, lanes 1–9). Activation of Tyk2 in response to IFN- α , therefore, appears dependent on the presence of JAK1. A similar dependence of JAK2 phosphorylation on the presence of JAK1 was observed (Fig. 6Ca). In wild-type cells JAK2 is phosphorylated

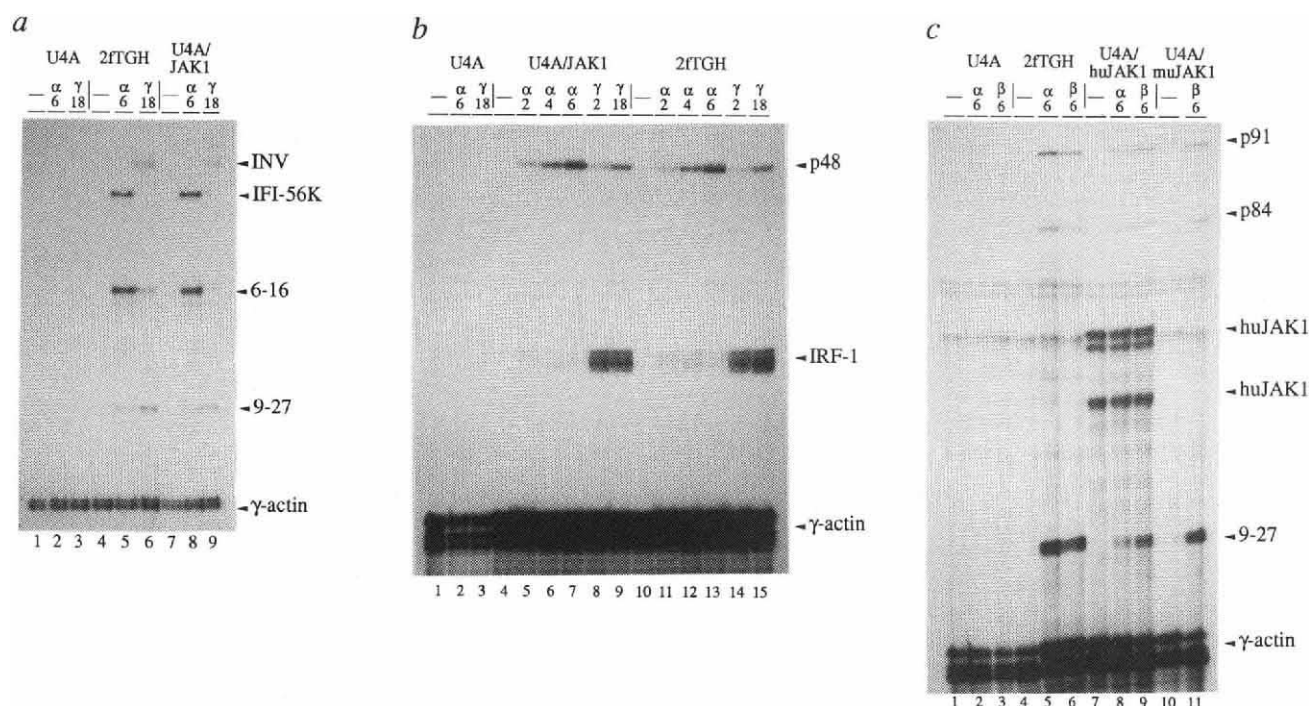


FIG. 4 Restoration of the transcriptional response to IFNs in U4A/JAK1 transfectants. Expression of mRNAs in response to IFN- α or - γ in 2fTGH, mutant U4A and U4A/JAK1 transfectant cells was monitored by RNase protection with γ -actin serving as an internal loading control. The following IFN-inducible mRNAs were analysed and listed according to their appearance from top to bottom of the autoradiographs: a, invariant chain of human class II HLAs (INV), IFI56K, 6-16, 9-27; b, p48, IRF-1; c, p91, p84 and 9-27. The times (in h) of treatment with 500 IU ml⁻¹ of either IFN- α , - β (in c) or - γ are indicated above the individual lanes. Recombinant human IFN- β _{ser} (3.6×10^8 IU mg⁻¹ protein) was from G.J. Williams, Berlex Laboratories, Alameda, USA. In c, human JAK1 and

mRNA was also assayed. The faster migrating protected JAK1 fragments were characteristic of the particular JAK1 probe (Fig. 1a) used and were consistently seen with high levels of JAK1 mRNA. The ribonuclease protection assay and the 6-16, 9-27, p91, p84, p48, IFI-56K, IRF-1 and γ -actin probes have been described previously¹⁴. The JAK1 probe was constructed by subcloning the HindIII–BglII fragment (nt 2,578 to 2,819) of the human JAK1 cDNA²⁷ into the pGEM4 vector (Promega). The INV probe consists of the PstI fragment (nt 1 to 308) of the invariant γ -chain of human class II HLAs⁵⁰ in pGEM4 (Promega). The human JAK1 protection probe does not protect the transfected murine JAK1 mRNA (lanes 10 and 11).

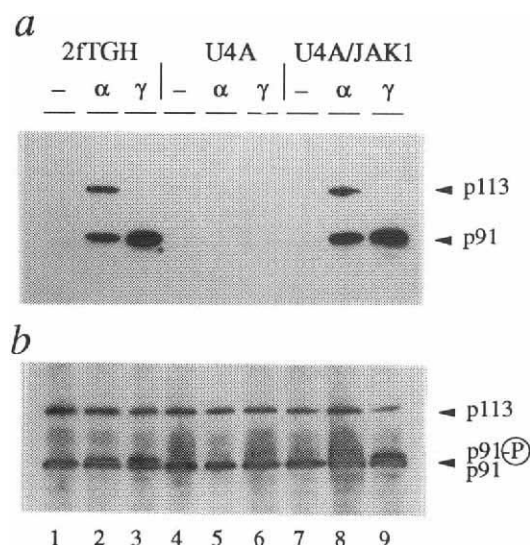


FIG. 5 Tyrosine phosphorylation of p113 and p91 in response to IFN- α or - γ in parental 2fTGH and mutant U4A cells and in U4A/JAK1 transfectants. Whole-cell lysates from IFN-treated or control cells were immunoprecipitated with antisera to p91 and p113^{4,6,7} and the immune precipitates were analysed by SDS-PAGE (7%) and western transfer using a, a mixture of PY20 (ICN) and 4G10 (UBI) antibodies to phosphotyrosine and, after stripping (in 0.1 M glycine buffer pH 2.5 for > 1 h and neutralization in 1 M Tris-Cl pH 8.0), b, a mixture of antibodies to p91 and p113. Treatment of cells with 500 IU ml⁻¹ of IFN- α or - γ , as indicated, was for 15 min at 37 °C.

in response to IFN- γ (Fig. 6Ca, lane 2) but not IFN- α . No such phosphorylation is observed in mutant U4A (Fig. 6Ca, lane 4) but it is restored in the U4A/JAK1 transfectants (Fig. 6Ca, lane 6).

Tyrosine phosphorylation of JAK1 is dependent on Tyk2 or JAK2. Having established that the IFN-mediated phosphorylation of Tyk2 and JAK2 is dependent on JAK1, performance of similar experiments with mutants U1D and γ 1A established a reciprocal dependence of JAK1 phosphorylation in response to IFN- α or - γ on Tyk2 and JAK2, respectively. Mutants in complementation group U1 lack Tyk2 and an IFN- α response, but retain an IFN- γ response^{32,33}. In such mutants there was no phosphorylation of JAK1 in response to IFN- α (Fig. 6D, lane 5) but phosphorylation in response to IFN- γ was retained (Fig. 6D, lane 6). (Mutant U1D was used in the experiment in 6D but similar results have been obtained with mutant U1A; G.B. and M.M., data not shown). Mutant γ 1A lacks activatable JAK2 and an IFN- γ response, but retains an IFN- α response (D. Watling *et al.*, accompanying paper⁵¹). In this JAK2-negative mutant phosphorylation of JAK1 is not observed in response to IFN- γ (Fig. 6E, lane 3), but is retained in response to IFN- α (Fig. 6E, lane 2). Phosphorylation of JAK1 in 2C4 cells, the parental line from which mutant γ 1A was derived, was

observed in response to both IFN- α and - γ (Fig. 6E, lanes 4–6) as is the case for the wild-type 2fTGH cells used throughout the remainder of these experiments (Fig. 6D, lanes 1–3).

Discussion

Mutant U4A cells lack the protein tyrosine kinase JAK1 (Figs 1 and 2). They express a truncated JAK1 mRNA corresponding to a 3' fragment plus additional 5' and/or 3' sequences not covered by the available cDNAs (Fig. 2a–d). Southern analysis failed to detect any major deletion or rearrangement in the corresponding gene (Fig. 2e). The results, therefore, suggest a point mutation, or small deletion or rearrangement in the JAK1 gene leading to deviant transcriptional initiation and/or mRNA processing. An independent mutation affecting either of these latter two processes cannot, however, be formally excluded.

Transfection of U4A cells with JAK1 cDNA expression constructs resulted in restoration of the IFN- α/β and IFN- γ response of all IFN-inducible genes tested; the mRNA expression levels and kinetics of induction of the genes analysed were identical in U4A/JAK1 transfectants and parental 2fTGH cells (Fig. 4). Consistent with these results, the activation by IFN-dependent tyrosine phosphorylation of known primary transcription factors was appropriately restored in

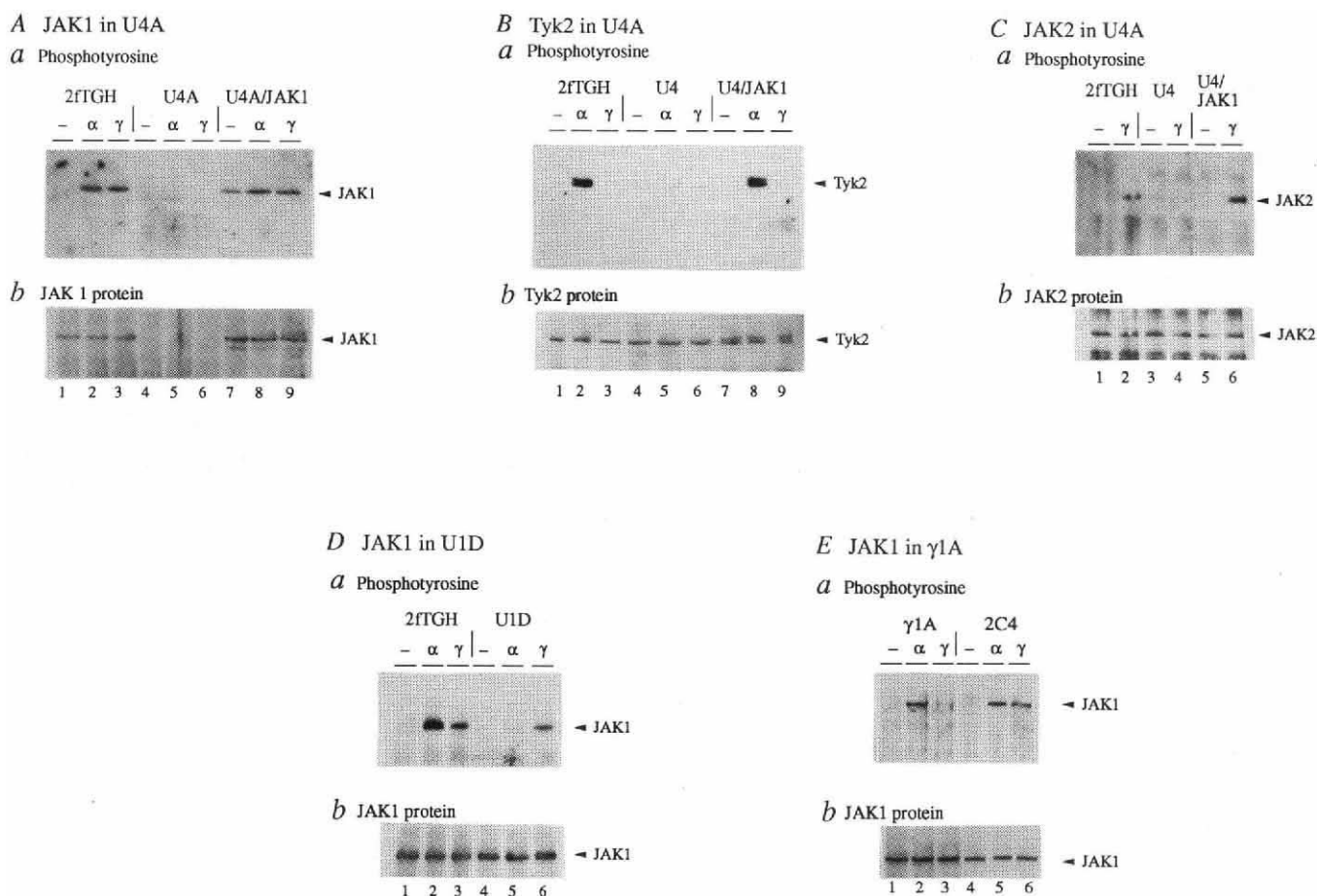


FIG. 6 Interdependence of the IFN response of JAK1, JAK2 and Tyk2. A–C, Tyrosine phosphorylation of A, JAK1, B, Tyk2 and C, JAK2 in response to IFN- α or - γ in JAK1-negative mutant U4A cells. D, E, Tyrosine phosphorylation of JAK1 in mutant U1D which lacks Tyk2 (D) and in mutant γ 1A which lacks JAK2 (E). In each case data for the corresponding wild-type cells are included as positive controls. Whole-cell lysates (as in Fig. 1) were immunoprecipitated with rabbit polyclonal antisera to JAK1²⁷ (A, D, E), Tyk2 (G.B., L. Velazquez, M. Scorogna, M. Fellous and S.P., unpublished results) (B) and JAK2³⁰ (C). The immunoprecipitates were analysed by SDS-PAGE (7%) and immunoblotting with

(upper panel, Aa–Ea) a mixture of antibodies to phosphotyrosine (as in Fig. 5) and, after stripping (lower panel, Ab–Eb) antisera to JAK1 (Ab, Db, Eb) or Tyk2 (Bb) or JAK2 (Cb). Treatment of cells with 500 IU ml⁻¹ IFN- α or - γ , as indicated, was for 15 min at 37 °C. Mutant U1D was isolated after 5 rounds of frame-shift mutagenesis of parental 2fTGH cells as described previously³⁴ and characterized by complementation analysis, ribonuclease protection assays and western analysis (M.M. and I.M.K., unpublished results). Parental 2C4 and mutant γ 1A cells are described by Watling *et al.* (accompanying paper⁵¹).

response to either type of IFN in cells complemented with JAK1 (Fig. 5).

JAK1 is rapidly phosphorylated on tyrosine after IFN- α / β and IFN- γ treatment (Fig. 6A). Such phosphorylation is not peculiar to 2fTGH cells and has been seen in a number of human and murine cell lines. Activation of JAK1 also occurs in response to IFN treatment. In *in vitro* kinase assays, presumptive auto-phosphorylation of JAK1 is observed in immune precipitates of JAK1 from cells pretreated with IFN- α or - γ but not from control cells (O.S., J.N.I., J. Schlessinger and D. E. Levy, manuscript in preparation; and D.G. and I.M.K., data not shown).

Experiments with a kinase-inactive mutant of JAK1 will be required to exclude the possibility that JAK1 protein, rather than its kinase activity, is all that is required to complement the defect in U4A cells, although this seems unlikely. The presence of a common substrate, p91, and of a common protein tyrosine kinase, JAK1, in the IFN- α and - γ response pathways makes it tempting to speculate that JAK1 might directly phosphorylate p91, that Tyk2 and JAK2 might function upstream of JAK1 and that p113, being unique to the IFN- α response, might be phosphorylated by Tyk2. But neither p113 nor Tyk2 or JAK2 is phosphorylated in response to the appropriate IFN in the absence of JAK1 in U4A cells (Fig. 5, 6Ba and Ca). Conversely, in the reciprocal experiments, JAK1 is not phosphorylated in response to IFN- α in the absence of Tyk2 in mutant U1D cells (Fig. 6D), or in response to IFN- γ in the absence of JAK2 in mutant γ 1A cells (Fig. 6E). JAK1 can, therefore, neither be placed upstream nor downstream of JAK2 and Tyk2 on the basis of these experiments. Similarly, the lack of phosphorylation of p113 in response to IFN- α in U4A cells (Fig. 5 lane 5) may result indirectly from the failure to activate Tyk2 or directly from the absence of JAK1.

One possible explanation for this interdependence of kinase activation is that, in response to IFN treatment, JAK2 and JAK1 or Tyk2 and JAK1 may be juxtaposed on receptor dimerization and cross-phosphorylate/activate. Alternatively, Tyk2 and JAK2 may indeed function upstream of JAK1, but fail to do so in mutant U4A because appropriate kinase-receptor complexes

are not formed in the absence of JAK1 protein. In this connection a variety of non-receptor protein tyrosine kinases (reviewed in ref. 37) are physically associated with cell membrane receptors^{38,40} and in some instances the biological significance of such interactions in signal transduction events has been demonstrated (reviewed in ref. 41). It has already been established that Tyk2 is required for the correct formation of the IFN- α receptor^{32,33} and the failure to activate JAK1 in U1 mutants may be a reflection of this. It is likely that there is an additional requirement for JAK1 for correct formation of the IFN- α receptor in that IFN- α binding is reduced in mutant U4A but restored in the U4A/JAK1 transfectants (J.B., M.M., I.M.K., data not shown). In addition, cell-free activation of the α -subunit of ISGF3 by IFN- α has been shown to be membrane-associated (ref. 42 and D.G. and I.M.K., unpublished results). Taken together, the data are consistent with a requirement for the presence of JAK family proteins for correct receptor assembly and with a model involving activation of transcription factors in receptor-associated complexes at the cell membrane in response to IFNs, as first suggested in ref. 43. Such complexes would now be postulated to include, in addition to classical trans-membrane receptor polypeptides, appropriate JAK family members and primary transcription factor components. An attractive aspect of this model is that the postulated inclusion of specific transcription factor polypeptides in the receptor complex (for example, p113 in the case of the IFN- α pathway), provides a mechanism for introducing specificity into pathways using common components.

Whether or not such a model proves correct, the availability of mutants defective in the JAK family kinases and in the transcription factor p91 will provide a suitable background for structure/function analyses of these components and of their potential interactions with each other and the IFN receptors. In addition, accepting the increasing evidence for the involvement of known, or immunologically crossreacting, members of the JAK family of protein tyrosine kinases and of p91, or p91-related proteins in other response pathways⁴⁴⁻⁴⁸, the introduction of alternative receptors into the mutant cells may considerably expand their value in analyses of this type. □

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Structure of the radio remnant of supernova 1987A

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SUPERNOVA 1987A in the Large Magellanic Cloud, the closest known supernova for 400 years, offers unprecedented opportunities for the detailed study of the evolution of a supernova at all wavelengths. The radio remnant of SN1987A was detected in July 1990 (ref. 1), since when it has steadily brightened at all radio frequencies². Its present brightness and size are now sufficient for its structure to be resolved. Here, we present high-resolution images of the remnant at a frequency of 8.8 GHz, which reveal a spherical, shell-like structure with a radius of 0.6 arcsec (4×10^{17} cm, assuming a distance of 50 kpc) and an additional component that is aligned with the optical ring (of somewhat larger radius) imaged by the Hubble Space Telescope³. We suggest that this alignment arises from an interaction between the expanding shock wave and dense clouds sheared from the ring. The mean expansion velocity of the supernova shock front, as measured from its current radio size, is $\sim 30,000$ km s⁻¹. Observations made over 600 days suggest, however, that either the remnant is rapidly changing shape or that the expansion velocity is decreasing more rapidly than expected.

Following an initial radio outburst⁴, SN1987A remained quiescent at radio frequencies for about two years. In July 1990, the source again became a radio emitter^{1,2}. Since that time it has been monitored regularly by the Australia Telescope Compact Array (ATCA)⁵ at frequencies from 1.4 to 8.9 GHz, showing a

more or less steady increase of flux density to its present (April 1993) level of 5 mJy at 8.6 GHz. The radio spectrum suggests that the radio emission is synchrotron radiation produced by relativistic electrons accelerated at the shock front^{6,7}.

The continued increase in flux density is different from the class of radio supernovae studied by Weiler and collaborators⁸. Radio supernovae show an initial rise in flux density, but after 10–1,000 days the overall flux density suffers a power-law decline⁹. Moreover, radio supernovae are much more intrinsically luminous than SN1987A (10^6 times more luminous in the case of SN1986J). The reason for this difference appears to be that radio supernovae have red supergiant progenitors which have massive outflows, or winds. The interaction of the shock front with this wind produces copious radio emission. On the other hand, the progenitor of SN1987A, Sk-69°202, evolved from a red supergiant to a blue supergiant just 2×10^4 yr before the explosion¹⁰. Such stars have fast tenuous winds which produce little radio emission when hit by a shock. This wind also appears responsible for sweeping away the former red supergiant wind into an equatorial ring³ of radius $\sim 6 \times 10^{17}$ cm. Therefore, the initial radio outburst from SN1987A (ref. 4) is apparently the analogue of the radio supernova phase, whereas the radio emission we are now seeing may well be the initial development of a classical supernova remnant similar to, for example, Cassiopeia A.

ATCA observations of SN1987A were made on 21 October 1992 (antenna configuration 6C), and on 4 and 5 January 1993 (antenna configuration 6A) simultaneously at two frequencies, 8.64 and 8.90 GHz. The observing bandwidth was 128 MHz for each frequency and all Stokes parameters were recorded. The integration time was nominally 12 h for each observation. The data were handled using conventional editing and calibration procedures, without self-calibration. A small amount of data obtained in periods of poor atmospheric phase stability was rejected. Imaging was done with a maximum entropy procedure^{11,12}, using a 0.4 arcsec (2.9×10^{17} cm) diameter gaussian restoring beam which is smaller than the conventional diffraction-limited beam of 0.85 arcsec. This procedure is justified by the high signal-to-noise ratio achieved by the long inte-

FIG. 1 Australia Telescope Compact Array images of the radio remnant of supernova 1987A at 8.8 GHz: a, diffraction-limited image using maximum entropy and a gaussian restoring beam of 0.85 arcsec FWHP (1992 October and 1993 January data); b, maximum entropy model image for 1992 October data; c, maximum entropy model image for 1993 January data; d, maximum entropy model image for combined 1992 October and 1993 January data; e, high-resolution image formed by convolving model (d) with a gaussian restoring beam of 0.4 arcsec FWHP and folding in the unscaled image residuals. Total flux density is 4.8 mJy. Image (f) is the Hubble Space Telescope [O III] image³ of the same region, showing the circumstellar ring to be only slightly larger than the size of the expanding radio remnant. Images were also made using a variety of other image deconvolution algorithms (Gerchberg-Saxon, Maximum Entropy, CLEAN and direct algebraic inversion) and produced results similar to or consistent with the images presented here. Also, simulations under conditions of identical signal-to-noise ratio and visibility coverage suggest that limb-brightened (or limb-darkened) models can be reliably recovered at 0.5 arcsec image resolution as in e. At this resolution, the total flux density contribution of spurious compact components arising from thermal noise is <5%.

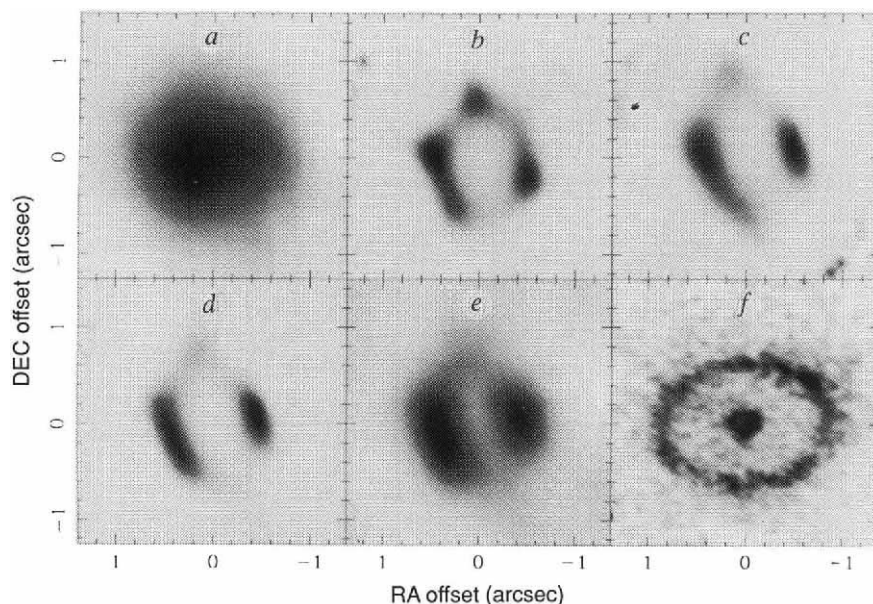
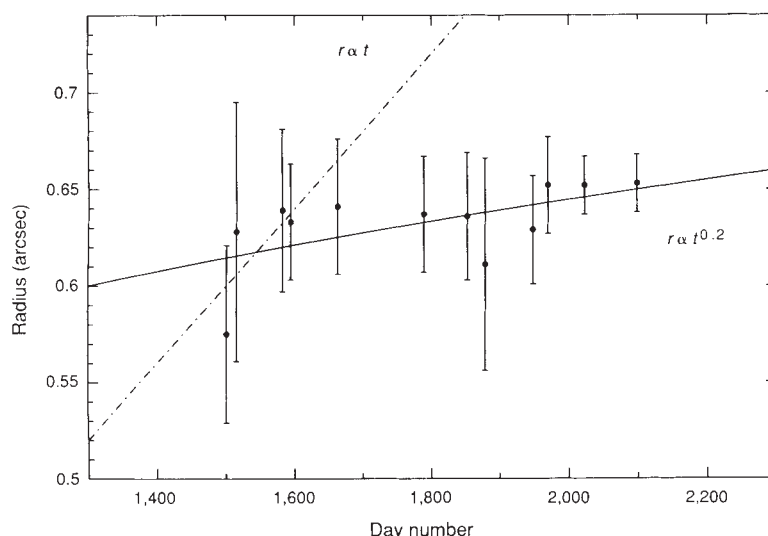


FIG. 2 Shell radius plotted as a function of time since the supernova explosion. The data are fitted by a power law $r \propto t^{0.2}$. The radii are based on model fits to the visibility data, on the assumption that emission is dominated by a thin transparent shell.



gration times and by the fact that, just before the October 1992 observations, the 8.8-GHz system temperature was halved to 30 K on the 6-km antenna (which is associated with all baselines in the 3 to 6 km range). Figure 1 shows the diffraction-limited image for the combined October 1992 and January 1993 data (no discernible structure; Fig. 1a); the maximum entropy model image for the October 1992 data (Fig. 1b); the maximum entropy model image for the January 1993 data (Fig. 1c), which agrees well with the October 1992 model image; the maximum entropy model image for the combined data (Fig. 1d); and the final image made by applying the 0.4 arcsec restoring beam to the combined model, and folding in the image residuals (Fig. 1e). (A high-resolution optical image is shown in Fig. 1f for comparison.) The resolution of the final restored image (Fig. 1e) depends on position in the image (the maximum entropy extrapolation depends on signal-to-noise) as well as the diameter of the restoring beam but lies in the range ~ 0.5 – 0.6 arcsec. The model images (Fig. 1b–d) and the final image (Fig. 1e) show a limb-brightened structure of radius 0.45 arcsec. This is consistent with the optically thin, synchrotron-emitting shell expected from interaction of the outer shock wave with the circumstellar medium. There is also an apparent increase in brightness at the eastern and western sides of the shell, a result consistent with earlier, more tentative, indications of ellipticity in the remnant^{2,13}. Flux-density measurements indicate that up to 50% of the total 8.8-GHz flux density could be located in these regions (including the smooth-shell component). This is consistent with recent 1.4-GHz very-long-baseline interferometry (VLBI) upper limits (W. Wilson, private communication) if the bright equatorial regions are extended at the 0.3 arcsec level.

Direct fits to the visibility data for a range of simple *a priori* models also gave results consistent with models dominated by a thin spherical shell rather than models with central brightening. The formal fit to a transparent, optically thin, infinitely thin shell model¹³ gives an angular radius of 0.65 ± 0.01 arcsec for the combined data. This is in good agreement with the smaller radius implied from the image when account is taken of image resolution.

The stability and excellent error estimation of the visibility modelling procedure makes it useful for data sets of much lower signal-to-noise ratio than the present data set. We therefore extend previous modelling¹³ of the 8.8-GHz observations of SN1987A to include all ATCA observations taken after the 6-km antenna was commissioned in April 1991, covering a period of 600 days. To maximize accuracy, we used only observations with full 12-h coverage. Figure 2 shows that, using the thin-shell model, the present increase of radius with time is low (and barely significant), 21 ± 10 mas yr⁻¹ ($4,800 \pm 2,300$ km s⁻¹). For com-

parison, the mean expansion rate required to explain the measured size around day 1,500 is 150 ± 5 mas yr⁻¹ ($34,600 \pm 1,200$ km s⁻¹), as shown by the broken line in Fig. 2. Therefore, before or during the onset of radio emission, there was substantial apparent deceleration of the shock front in the intervening medium. A power-law fit between April 1991 and January 1993 yields $(r/r_0) = (t/1,800 \text{ days})^m$, with $r_0 = (4.60 \pm 0.04) \times 10^{17}$ cm and expansion index $m = 0.17 \pm 0.08$. Similar results are obtained with other simple models (such as a thin shell with two hotspots).

The expansion index is well below that predicted⁹ ($m \approx 0.8$ – 0.9) for a shock front propagating through a normal stellar wind and lower than that directly observed in the case of VLBI angular diameter measurements¹⁴ of SN1979C ($m = 1.03 \pm 0.15$). One possibility for the high deceleration implied for the SN1987A shock is that it has now left the free wind region and entered the relatively constant-density shocked-wind region predicted in models¹⁵ of fast stellar winds ploughing into stationary or slowly moving media. However, only gentle deceleration ($m \approx 0.6$) will result unless the shock has almost reached the contact discontinuity, or outer edge of the blue supergiant wind, where the density can increase sharply^{16,17}. The fact that the radio shell is still only at 76% of the radius of the 0.85 arcsec circumstellar ring³, which delineates the contact discontinuity, suggests that the shock has not yet reached this boundary.

Another possibility is suggested by previous monitoring of the 4.8-GHz radio emission which has indicated variability on a short timescale². If the shock wave is propagating through an inhomogeneous medium consisting of discrete high-density clouds, the energy transferred from the shock front into internal cloud energy ($\int P dV$) can be substantial. This may contribute to the deceleration of the shock front without large increases in density¹⁸. If the radio emission is also associated with these high-density clouds, it is then possible that the outer shock front has swept some distance beyond the radius of the radio shell. But, the continued increase in radio power argues against a radio shell not closely associated with the shock front.

A final, more attractive, possibility is that the radio remnant is getting thicker with time. This would bias the model fits shown in Fig. 2, which are only valid for an emission shell of small thickness. For example, model fits to a thick shell, with a ratio of outer radius to inner radius of 1.25 (volume-filling factor, 0.5), would give outer angular radii $\sim 10\%$ higher than those plotted in Fig. 1. If the remnant evolved from a thin shell at day 1,500 to a thick shell at 2,100, the derived expansion rate would be ~ 60 mas yr⁻¹ ($14,000$ km s⁻¹), which is more in line with the expected change in expansion velocity.

The strong 'hotspots' in the radio image in Fig. 1e are at a

similar position angle to that of the circumstellar ring ($\sim 80^\circ$). This suggests equatorial density enhancements perhaps arising from instabilities at the interface between the red and blue supergiant winds. Eventually, the increasing density will slow the shock front in the equatorial directions, if this is not already happening. Continued observations of SN1987A over the next few years may therefore reveal a larger remnant size along the less dense polar direction, possibly resulting in a remnant shape similar to the classical barrel-like supernova remnants¹⁹.

Continued observations may also reveal the relationship between the class of radio supernovae and the remnants they eventually form. When will we be able to observe the strong radio and X-ray emission and the shocked ultraviolet and optical

lines predicted to occur when the shock front reaches the circumstellar ring? An earlier predicted date²⁰ of 2002 is based on a mean velocity $\sim 10^4 \text{ km s}^{-1}$. But, given the higher *mean* velocities inferred from the radio results, this may be a considerable overestimate. The presence of excess equatorial emission already shows that interaction of the shock with the low-density inner part of the circumstellar torus, or high-density clouds sheared from this torus, has begun. Direct imaging, perhaps at higher frequencies, over a period of a few years will enable us to resolve the issue of the nebular expansion rate and help predict the date for interaction with the high-density torus with greater accuracy. $\square$

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Highly anisotropic light emission from laser breakdown of microparticles in water

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LASER breakdown, in which a substance is transformed explosively to a plasma by a focused pulsed laser beam, has been much studied^{1–8}. Light emission from the plasma formed by breakdown of liquid media or suspended microparticles is generally observed lateral to the laser beam, and can be used for spectrochemical analysis^{4,8,9}. Here we report the observation of highly anisotropic emission of hydrogen Balmer- α ($H\alpha$) radiation from the laser breakdown of polystyrene particles in water. The emission was strong and spectrally narrow in the forward and backward directions (with respect to the beam), and weak and broad in others. The incorporation of a single-mirror cavity in the optical arrangement and the observed nonlinear dependence of intensity on the focal length of the focusing lens suggest that the plasma may induce gain in the emitted radiation. The exact mechanism of the anisotropic emission is not yet known.

The plasma emission from laser breakdown of polystyrene microparticles in water was observed from both forward and lateral directions through an optical cut-off filter (Fig. 1a). Microparticles (0.3- μm diameter) of polystyrene were dispersed in ultra-pure water and poured into a quartz cell. The particle number density was $6.7 \times 10^5 \text{ cm}^{-3}$, at which the particle number expectation in the focal region was >1 for every laser shot⁷. With the optical power density employed, particle laser breakdown could be induced in the focal region (Fig. 1b), because the laser breakdown threshold of the particle is lower than that

of the liquid medium⁶. Several particles may be broken-down simultaneously at this particle number density.

We observed clear red points in the projection spot of the plasma emission in the forward direction when a screen was placed perpendicular to the beam axis in front of the spectrometer (Fig. 1a). Figure 2 shows photographs of the pumping beam and the plasma emission projected on the screen. These photographs were of single-event shots, in which Fig. 2a shows the pumping beam when its power density was decreased so as not to induce particle breakdown, and Fig. 2b shows the instant of breakdown in which the white part of the picture is plasma emission and the peripheral green part is the pumping beam scattered by the plasma and optical components. These photographs were taken without using an optical filter. But when the filter was inserted to reduce the plasma emission and pumping beam, distinct red spots appeared in the region of the plasma emission (Fig. 2c). A typical measured spectrum of this strong directive red emission is shown in Fig. 2d (labelled $\theta = 0^\circ$; θ is defined in Fig. 1a) along with that measured from the lateral direction ($\theta = 90^\circ$). In comparing these spectra, the $H\alpha$ line at 656.3 nm was seen to be strongly anisotropic. The sharp peak of the $H\alpha$ line could not be seen from the lateral direction when the sensitivity of the detection system was identical to that used for the measurement from the forward direction. A broad peak was observed, however, when the sensitivity was increased 40 times. Direct comparison of the forward and laterally directed spectra indicated the emission of the former was 42 times stronger and 80 times narrower than the emission of the latter. The same anisotropy of the $H\alpha$ line was also observed in the 180° backward direction.

Electron density and temperature of the plasma, which were estimated from repeated measurements of the $H\alpha$ emission from the lateral direction, were $\sim 10^{17}$ – 10^{18} cm^{-3} and 2–4 eV, respectively. These values, although dominated by conditions very early in the plasma expansion, agreed with those of other reports on under-water laser breakdown^{8,9}. As discussed later, the broad peaks at 648.8 and 650.6 nm seen in the forward-direction spectrum could be assigned to Raman scattering of the pumping beam by a symmetric stretching vibration of water molecules in the plasma. They appeared only when breakdown occurred; hence they may be the stimulated Raman scattering (SRS)

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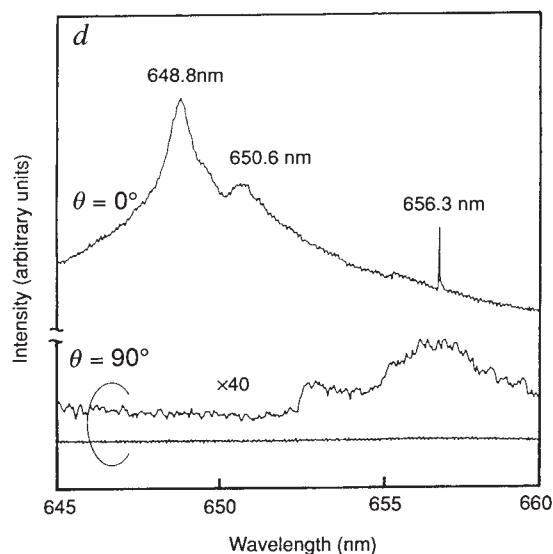
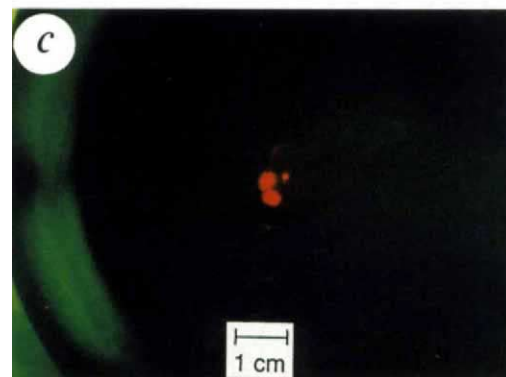
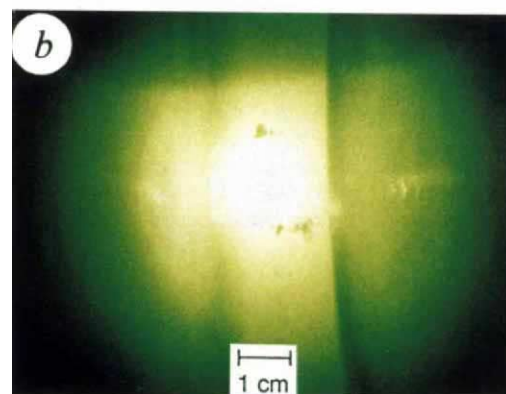
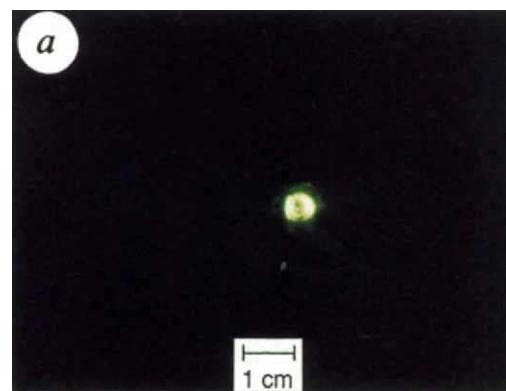
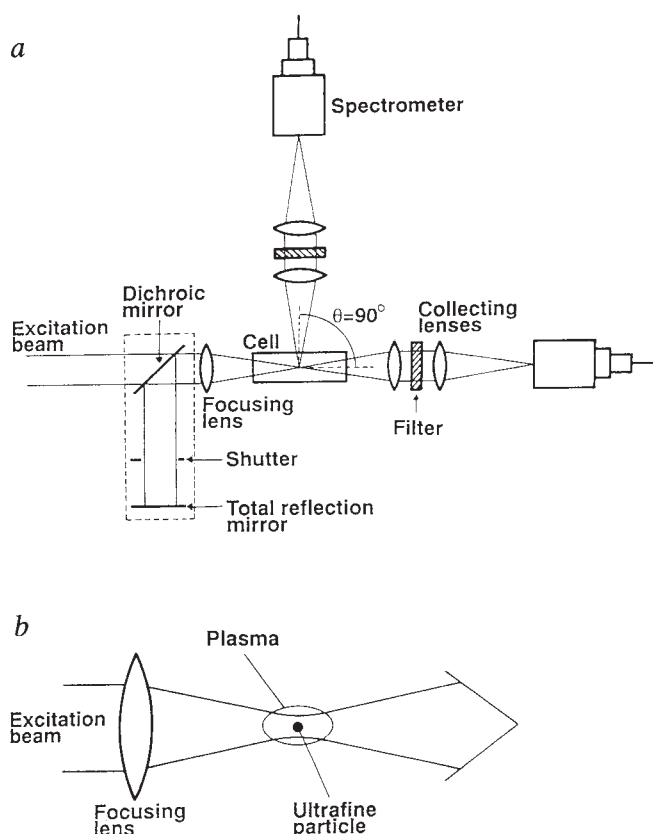


FIG. 1 Schematic illustration of the experimental arrangement (a), and a symbolic representation of a particle laser breakdown phenomenon (b). The second harmonic of a YAG laser ($\lambda = 532$ nm) was focused in the water. The laser power was 23 mJ per pulse and the pulse duration was 8 ns. The fluctuation of the laser power, that is, long term stability, was within 5%. The beam diameter was 9 mm and the focal length of the focusing lens was 80 mm, hence the minimum beam waist at the focal region was estimated as $4.5 \mu\text{m}$ from the calculation for a gaussian beam of the same size, and the optical power density was $1.8 \times 10^{13} \text{ W cm}^{-2}$ at most. But the beam profile was not really a gaussian shape, so that the power density should be lower than the estimated value. The repetition ratio of the excitation laser pulse was 10 pulses s^{-1} . An optical filter (cut-off wavelength 560 nm) was placed in front of the detection system to reduce the strong background of the pumping beam and plasma emission. The quartz cell was 10 mm wide, 50 mm long in the optical axial direction and 40 mm high. The size of the $0.3\text{-}\mu\text{m}$ polystyrene microparticles was well controlled, and their coefficient of variance was within 0.3%. As the specific gravity of the particle was 1.05, such microparticles were suspended well in the water.

FIG. 2 Photographs of the forward projection of the pumping beam spot (a), laser breakdown plasma emission (b), the laser breakdown plasma emission through optical cut-off filter (c), and spectra (d) of the red spots of c and the plasma emission radiated to the lateral direction of the pumping beam. Photographs b and c were obtained under identical conditions. Plasma emission spectra were measured by a $f = 250$ mm and $1,800 \text{ lines mm}^{-1}$ grating spectrometer and detected by an optical multichannel detector using a microchannel plate with a spatial resolution of $25 \mu\text{m}$. The total spectral resolution of this system was calculated as 0.047 nm . In practical use, however, the inferior resolution (0.1 nm equivalent to two channels) should be taken into consideration. It was confirmed that the optical filter and the other optical components were non-fluorescent in the visible region for visible and ultraviolet excitation.

observed previously in laser breakdown phenomena^{3,10}. The red spots of Fig. 2c might be due to this SRS, considering the peak area.

Time profiles of the plasma emission were measured with a spectrometer and streak tube detection system at three wavelengths; the H α line (Fig. 3a), the continuum (at 660.0 nm) (Fig. 3b) and that of the pumping beam (Fig. 3c). Peak B in Fig. 3a, which was obviously distinguishable from noise, was observed at ~ 20 ns after irradiation by the pumping beam (peak D in Fig. 3c). The delay time fluctuated between 20 and 30 ns in each breakdown event. Compared with the temporal profile of the continuum (Fig. 3b), peak A was considered to be ordinary plasma emission and the foot of the Raman band shown in Fig. 2d. The delayed emission corresponding to peak B was considered to be the anisotropic H α emission. Thus the H α line is neither electromagnetic noise nor shot noise. On the other hand, the atomic emissions of the laser-induced under-water plasma, which were observed from the lateral direction, always lasted through the plasma continuum emission and had a duration of ~ 0.1 μ s or longer, depending on the medium^{3,9}. Their spectra were broadened by the Stark effect, and the spectra shape depended on the momentarily changing electron density and temperature of plasma. Hence, direct comparison between the forward and lateral spectra without time resolution can not give a reliable estimation of the enhancement.

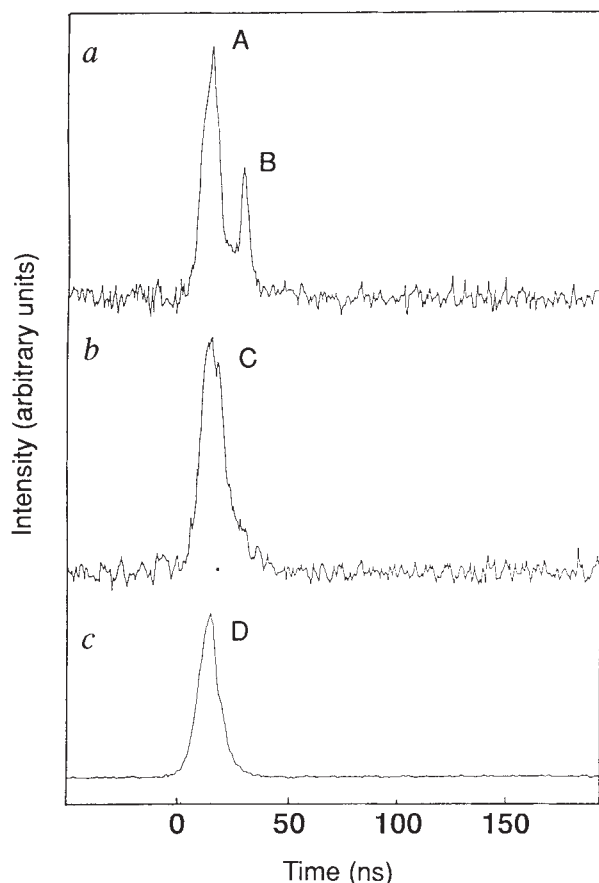


FIG. 3 Temporal profiles of the plasma emission at wavelengths of the H α line (a), the background plasma continuum part (660 nm) (b), and the pumping beam (c). These temporal profiles were measured by using a low-speed streak tube system having a resolution of the time axis of 0.59 ns per channel. The spectrometer placed before the streak tube was the same as used in the measurement of Fig. 2d. The spatial resolution of the image intensifier placed after the streak tube was 80 μ m, hence the practical spectral resolution of this system was about 0.3 nm. (The peaks labelled A, B, C and D are discussed in the text.)

We have studied the effect of incorporating a two-pass cavity and estimated the enhancement. A dichroic mirror and a total-reflection mirror (shown in Fig. 1a inside the broken lines) were added to the fundamental optical arrangement to construct a single mirror cavity. The emission radiated in the 180° backward direction was separated from the pumping-beam axis by the dichroic mirror, and reflected perpendicularly by the total-reflection mirror. It then re-entered the plasma via the dichroic mirror and the focusing lens of the pumping beam. If the plasma has gain, the light that re-enters and passes through the plasma will be more intense than the initial backward emission. Assuming the gain-length product is gL (g , gain coefficient; L , plasma length) and writing the intensities of the forward and backward emissions of the H α line as A and B , respectively, the total enhanced emission with this two-pass cavity is represented as $A + Be^{gL}$. The cavity could be actuated by opening the shutter. The optical path-length between the total-reflection mirror and the beam waist was set at 30 cm, so the time required for returning the emission to the plasma was 2 ns. Now, the duration of the anisotropic H α emission was ~ 5 ns as shown in its temporal profile in Fig. 3a. Hence, the returning time was sufficiently small compared to the period in which the plasma was surmised to work as a gain medium. The measured intensities of the H α emission were normalized by the value measured from the forward direction without actuating the cavity. When the cavity was actuated, the relative intensity of the H α emission was 4.6 (larger than the sum of the forward and backward emissions ($A + B$) of 3.0), and the effect of the cavity on the enhancement was confirmed. Furthermore, we calculated the enhancement e^{gL} and found that for the two-pass cavity system it was 1.8 with a focusing lens of focal length f of 80 mm, and up to 27.9 for $f = 200$ mm. This nonlinear dependence on the focal length was also considered to be evidence that the plasma acted as a gain medium. In addition, the plasma length during the anisotropic emission was estimated by using a dual-purpose framing or streak camera (50 ns exposure time and 10 ps temporal resolution in the respective modes), and found to be 200 μ m at most for a lens with $f = 80$ mm. The gain coefficient g under this condition was calculated as 40 cm^{-1} , although this value may still be an underestimate because of scattering on the plasma surface and other factors.

To confirm that the hydrogen in the gain medium comes from the polystyrene microparticle, generation incidence was measured for four combinations of medium and particle, that is, hydrogen-containing and non-hydrogen-containing species. The hydrogen-containing particle and medium were 0.3- μ m polystyrene and water, whereas the non-hydrogen-containing ones were 0.7- μ m silver particles and carbon tetrachloride. When the particle contained hydrogen, generation incidence of the anisotropic H α emission ($\sim 1/150$) was significantly higher than in the other cases ($\sim 1/1,000$), regardless of the medium. Therefore, the gain-medium hydrogen component originated not in the medium, but in the particle. When the medium was carbon tetrachloride, yellow rather than red spots were seen. The spectrum of the yellow spots agreed well with the Raman scattering of carbon tetrachloride¹⁰. Hence SRS was also confirmed, and it indicated that vaporized molecules of the medium existed in the plasma from very early in its formation.

The results reported here suggest a lasing-like action. In fact, the delay time of this highly anisotropic light emission agreed well with the value extrapolated from the calculated temporal profile of population inversion density in laser-induced recombining hydrogen plasma^{11,12}. However, some inconsistencies with theoretical studies^{11,13} appeared. Our estimated gain coefficient was larger than the theoretical prediction, and the plasma parameters were upper-limit values or just above the thermal limit of initial conditions of recombination lasing. One possible explanation is that the delayed H α emission occurred when the plasma had expanded and cooled enough to have significant gain and a narrow line. Also, the emission wavelength shifted

randomly in each breakdown event. The maximum shift was 2 nm, and it was too large for atomic emission, although we have confirmed an isotope effect on each shifted line from line broadening by superimposition of $D\alpha$ and $H\alpha$ lines, which will be reported elsewhere. $\square$

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Building one- and two-dimensional nanostructures by diffusion-controlled aggregation at surfaces

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THE formation of nanometre-scale surface structures by atomic manipulation with the scanning tunnelling microscope has opened up opportunities for creating new metastable states of matter atom by atom¹. The technique allows the fabrication of arbitrary structures, but its application may be limited by considerations of speed, as only one nanostructure can be built at a time. Here we describe the simultaneous formation of many densely packed nanostructures of various morphologies using diffusion-controlled aggregation on surfaces. By exploiting the dependence of the mobility of adsorbed atoms on substrate crystal face and temperature, we are able to grow linear, two-dimensional or tenuous fractal aggregates of nanometre dimensions. The high number density (10^{11} – 10^{14} cm⁻²) of these structures means that their physical and chemical properties can be easily measured with conventional surface spectroscopies.

The deposition of atoms onto a substrate is a non-equilibrium process; the system tries to restore equilibrium by forming aggregates². The adatoms migrate on the surface and when meeting each other they can form critical nuclei, which subsequently can grow to clusters and islands by attachment of further adatoms. Nucleation and growth are competing processes. The outcome of this competition is determined by the surface diffusion (r.m.s. surface area migrated by an adatom in unit time) and the deposition flux (atoms deposited per unit time and unit surface)². The diffusion of an adatom stops when it hits a critical nucleus or a stable aggregate and condenses there. With increasing coverage, the density of stable nuclei increases until a saturation density is reached. After this, impinging atoms condense solely at existing aggregates. At this stage they are migrating the

average distance Λ_a . The average adatom diffusion length is temperature (and flux)-dependent. At low temperatures Λ_a is small, a high density of stable nuclei is formed and small clusters grow. With increasing temperature Λ_a increases, the saturation island density decreases and larger aggregates can grow. The shape of these aggregates is determined both by the directional anisotropy of Λ_a and by the average diffusion length of atoms adsorbed at the perimeter of the aggregate Λ_l (refs 3, 4). At low temperatures $\Lambda_l \approx 0$, that is, atoms attaching to an aggregate stick where they hit (the usual assumption in diffusion limited aggregation (DLA) simulations³). At elevated temperatures, however, atoms adsorbed on the edge of the aggregate can continue to migrate around the perimeter, that is $\Lambda_l > 0$. Here we will demonstrate that by means of temperature control of Λ_a and Λ_l , the size and shape of the aggregates formed can be manipulated specifically on an atomic level.

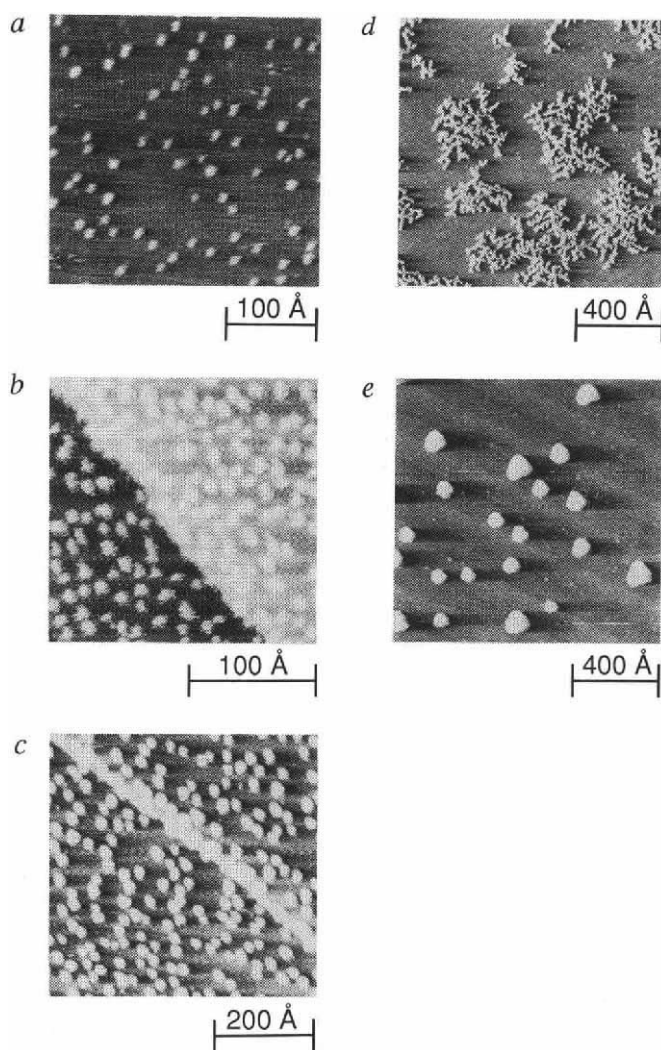


FIG. 1 Scanning tunnelling microscopy (STM) images of two-dimensional Ag aggregates grown on the Pt (111) surface. *a*, Ag dimers grown and imaged at 40 K. The total coverage of the surface (in monolayers, ML) by silver, $\theta_{Ag} = 0.012$ ML. *b* and *c*, Compact two-dimensional Ag clusters of average size $n = 6$ (*b*) and $n = 26$ (*c*) ($\theta_{Ag} = 0.12$ ML) synthesized by growing dimers at 40 K and successive annealing to 110 K and 150 K, respectively (these are also STM imaging temperatures). *d*, Large fractal Ag aggregates ($\theta_{Ag} = 0.12$ ML) grown and imaged at 110 K; the deposition flux was reduced here to 1 ML per 6×10^4 s. *e*, Two-dimensional equilibrium-shape Ag islands obtained by annealing dimers (such as shown in *a*) to a temperature of 280 K ($\theta_{Ag} = 0.12$ ML).

The experiments were performed with a variable-temperature scanning tunnelling microscope (STM) operating in ultra-high vacuum⁵. The accessible temperature range for *in situ* measurements is 25–600 K. The systems we have studied are Ag aggregates formed on the Pt (111) surface and Cu aggregates grown on Pd (110). The metal adsorbates were evaporated onto the substrates from molecular-beam-epitaxy Knudsen cells at rates of typically one monolayer per 900 s. All STM images have been acquired in a constant-current mode with tunnelling currents in the range 1.0–3.0 nA at sample voltages from –0.5 to –1.5 V.

At the very lowest temperatures, adatom migration is slow with respect to deposition resulting in small values of Λ_a (a few Å) and a high density of stable nuclei. An example of this regime is shown in Fig. 1a. Here 1.2% of a Ag monolayer has been deposited on the Pt (111) surface at 40 K. The migration barrier of Ag adatoms on Pt (111) being 140 ± 10 meV (ref. 5), the average diffusion length turns out to be only 9 Å at this temperature, giving rise to the exclusive aggregation of homogeneously distributed Ag dimers.

With increasing substrate temperature during deposition, Λ_a increases, the saturation island density decreases and larger clusters can grow (aggregates containing several atoms to thousands of atoms). The shape of these clusters, in the absence of directional anisotropies in Λ_a , is determined by the average diffusion length of atoms adsorbed at the perimeter of the aggregate, Λ_l . For small values of the perimeter diffusion length ($\Lambda_l \approx 0$ in DLA), the aggregates should grow in a ramified fashion, whereas for higher values the islands are expected to condense in compact shapes^{3,4}. An example in which the hindered perimeter diffusion leads to ramified growth is shown in Fig. 1d; large fractal Ag aggregates grow on the Pt (111) surface at 110 K. A detailed analysis reveals the expected mass-length scaling behaviour. The large aggregate size (~ 300 Å) has been tailored by reducing the flux to 1.7×10^{-5} monolayers per second, leading to a saturation island density of only $1.05 \times 10^{11} \text{ cm}^{-2}$ as expected⁶. Under these conditions the free adatom mobility is sufficiently high ($\Lambda_a = 200$ Å) while the edge diffusion is still frozen. For self-diffusion on face-centred cubic transition metals, it has been found that migration barriers for edge diffusion (the channels on the (331) and (113) surfaces) are ~ 3 –4 times larger than for (111) terrace diffusion⁷.

The equilibrium island shape is formed by a brief anneal of kinetically determined aggregates to temperatures > 250 K. The two-dimensional Ag islands shown in Fig. 1e, have been obtained by annealing dimers, such as those shown in Fig. 1a, to

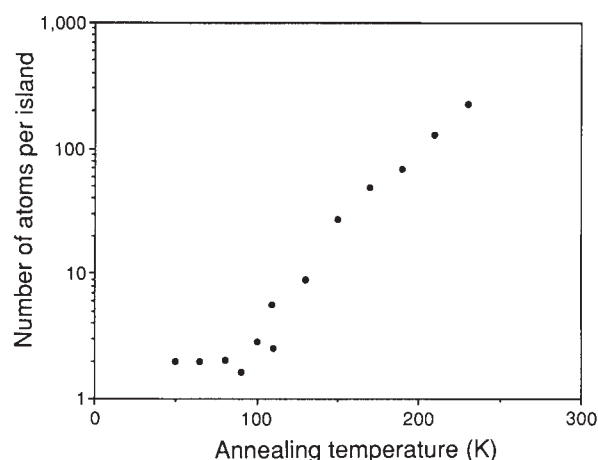


FIG. 2 Thermal stability of the Ag dimers synthesized by means of aggregation at 40 K. On annealing above 100 K, larger two-dimensional Ag clusters can be formed.

280 K. The observed hexagonal equilibrium shape with trigonal symmetry reflects the free energy ratio of the $\langle 1\bar{1}0 \rangle \{100\}$ and $\langle 1\bar{1}0 \rangle \{111\}$ ($\langle \text{direction} \rangle \{ \text{microfacet} \}$) island edges $\delta\{100\}/\delta\{111\} = 1.25$ (ref. 8).

Of particular importance is the intermediate range of cluster sizes, from a few atoms up to several hundred atoms. Many experimental and theoretical efforts are now exploring the evolution of physical and chemical properties as a function of the number of atoms involved⁹. The most dramatic effects are expected for small clusters with sizes up to several tens of atoms. Two-dimensional clusters of this size could in principle be nucleated on surfaces directly by diffusion-controlled aggregation, at an appropriate temperature, flux and coverage. According to nucleation theory⁶ the cluster-size distribution of these aggregates would, however, be rather broad. We have explored an alternative route that gives rise to narrow cluster-size distributions (Figs 1b, c, and Fig. 2). In the first step of this process; a homogeneous distribution of stable nuclei is grown on the Pt (111) surface. The clusters are formed in the second step by thermally activated coalescence of these nuclei. Two typical results are shown in Fig. 1b, c, where compact Ag clusters with an average number of atoms (n) of 6 and 26 have been aggre-

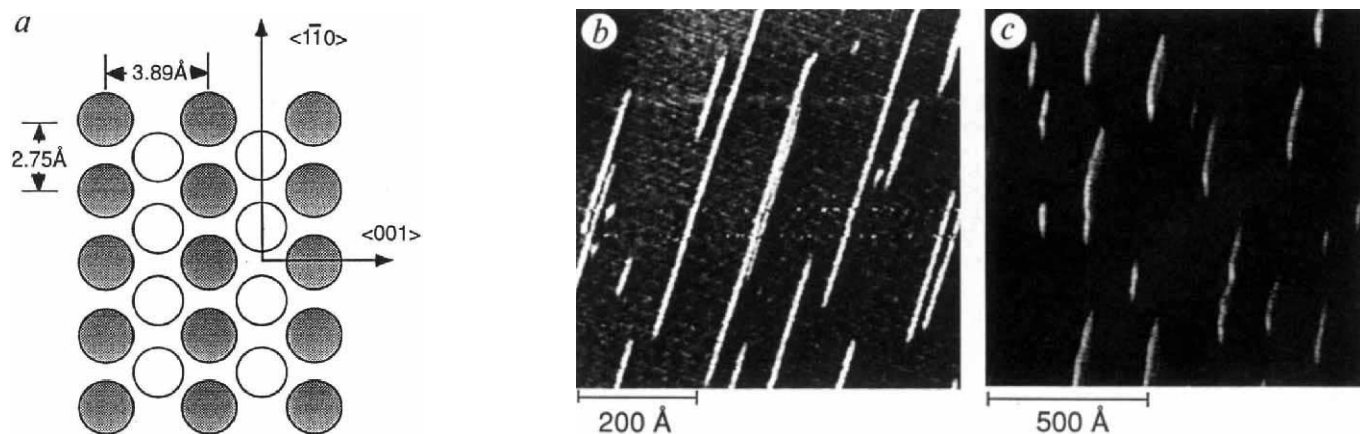


FIG. 3 Cu aggregates grown on the anisotropic Pd (110) surface. a, Surface geometry. b, STM image of monatomic Cu wires grown on the Pd (110) surface; the one-dimensional copper chains were grown and imaged at 300 K, the total coverage was $\theta_{\text{Cu}} = 0.05$ ML. c, The transition

from one-dimensional chains to two-dimensional islands upon growth at 350 K ($\theta_{\text{Cu}} = 0.1$ ML) demonstrates the kinetic origin of the monatomic wires.

gated via nucleation of dimers at 40 K and subsequent annealing to 110 K and 150 K respectively. The homogeneous spatial distribution and the narrow range of sizes are obvious in both STM images.

The diffusion barrier of the pre-nucleated dimers is twice as high as that of Ag adatoms¹⁰ and the dimers are stable over an extended temperature range (Fig. 2). On annealing the surface to temperatures >100 K, dimer diffusion and/or dissociation and subsequent adatom diffusion sets in, resulting in the formation of larger clusters by coalescence. The size of these clusters depends exponentially on the annealing temperature.

Diffusion-controlled aggregation can also be used to synthesize one-dimensional aggregates. One-dimensional systems have, indeed, been the focus of much interest because of their unique behaviour and the fact that certain problems can be solved exactly for these systems¹¹. The growth of one-dimensional aggregates makes use of the directional anisotropy of Λ_a (ref. 12). For surfaces with C_{2v} symmetry, there are two different migration barriers, representing two orthogonal directions. An example is the diffusion of Cu atoms on the Pd (110) surface, where the migration barriers are 0.76 and 0.51 eV for the orthogonal [001] and [110] directions, respectively¹³. The value of Λ_a [110] is thus larger than Λ_a [100] giving rise to a faster growth rate along the [110] channels. With decreasing deposition temperature, the ratio of the rate of diffusion along the close-packed direction to that in the perpendicular direction increases. It should therefore be possible to freeze out cross-channel diffusion along [100] while diffusion along the [110] channels is still sufficiently fast. Under such favourable conditions, exclusively one-dimensional chains of adatoms should grow along the [110] direction of the surface.

The experimental realization of such a situation is shown in Fig. 3b, in which Cu aggregates are grown and imaged on the Pd (110) surface at 300 K. At this temperature, the jump rate in the easy [110] direction is 10^4 times higher than that across the channel walls (assuming approximately equal prefactors¹⁴) and we observe the aggregation of long monatomic Cu chains with lengths up to several hundred Å. The average length of these one-dimensional Cu wires can be tailored, for example by varying the deposition temperature. Because diffusion along [100] is already negligible at 300 K, nucleation at temperatures <300 K will also result in the aggregation of one-dimensional chains, the average length of which will decrease with decreasing temperature (that is, decreasing Λ_a [110]). Whereas at 300 K for a total coverage of 0.05 ML the monatomic Cu chains consist of an average of 130 Cu atoms, at 180 K (at the same coverage) the average chain is composed of only 10 Cu atoms.

The monatomic Cu chains aggregated on the Pd (110) surface are metastable nanostructures. Upon a short anneal to, or direct growth at, 350 K, two-dimensional lens-shaped islands, several atomic rows wide, are formed. This is demonstrated in Fig. 3c, which shows the structure produced by growth at 350 K: two-dimensional Cu islands with an average aspect ratio of 15 are formed. The metastable character of the long one-dimensional Cu chains is not surprising. Field ion microscopy measurements^{15–17} and molecular dynamics calculations¹⁸ have revealed that in general the linear one-dimensional clusters constitute the equilibrium structure only at small sizes ($n < 10$). For Cu on Pd (110), however, recent molecular dynamics simulations¹⁹ reveal that at 0 K one-dimensional chains (which can be very long) are the energetically preferred structures. It appears to be the entropy gain associated with the transition from one to two dimensions that drives the Cu aggregates on Pd (110) to two-dimensional structures at elevated temperatures¹³.

This approach of diffusion-controlled aggregation for the synthesis of one- and two-dimensional matter is not restricted to the systems discussed, nor to metal aggregates on metal surfaces. Diffusion-controlled aggregation is applicable for all growth systems in the kinetic regime (including metals on semiconductors or insulators), and one should always be able to find a tempera-

ture window in which one- or two-dimensional aggregates (substrates with directionally anisotropic diffusion) can be produced by manipulating Λ_a and Λ_l . □

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Atomic-resolution chemical analysis using a scanning transmission electron microscope

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THE high angle elastic scattering of electrons in scanning transmission electron microscopy depends strongly on the atomic number Z , of the sample atoms, through the Z^2 dependence of the Rutherford scattering cross-section¹. The detection of scattered electrons at high angles and over a large angular range (75–150 milliradians) removes the coherent effects of diffraction, and the resulting incoherent image provides a compositional map of the sample with high atomic-number contrast¹. If a fine electron probe is used, and the sample is a crystalline material oriented along one of its principal axes, individual columns of atoms can be imaged in this way². Electrons scattered at low angles are not used in this detection scheme, and are thus available for simultaneous electron energy-loss spectroscopy³; in principle, this combination of techniques should allow the direct chemical analysis of single atomic columns in crystalline materials. Here we present electron energy-loss spectra from epitaxial interfaces between cobalt silicide and silicon, which confirm that atomic resolution can be achieved by this approach. The ability to correlate structure and chemistry with atomic resolution holds great promise for the detailed study of defects and interfaces.

The basis of obtaining electron energy-loss spectra (EELS) with atomic resolution is the high-resolution Z -contrast imaging technique in the scanning transmission electron microscope (STEM) (Fig. 1). Using the high-angle annular dark-field detector makes thermal diffuse scattering the dominant contribution to the detected intensity and allows us to consider each atom as scattering independently with a cross-section that approaches the Z^2 dependence on atomic number. For thin specimens, where there is no dynamical diffraction, the detected intensity will

therefore consist of a convolution of the probe profile and an object function which is strongly peaked at the atom sites. As the width of the object function is typically ~ 0.1 Å, this means that the spatial resolution is limited only by the 2.2-Å probe size of the STEM (VG Microscopes HB501 UX). In zone-axis orientations where the probe size is smaller than the atomic spacing, the individual atomic columns can therefore be excited individually and an atomic resolution compositional map of the specimen is generated. Experimental results show that this high resolution also holds for thicker specimens where dynamical diffraction would be expected to significantly broaden the probe². This is due to the local nature of the high-angle scattering events resulting in a preferential scattering from *s*-type Bloch states. Dynamical diffraction then manifests itself simply as a columnar channelling effect which scales the scattering cross-section according to thickness^{2,4,5}. Differences in intensity due to channelling effects are always less than those due to compositional changes, so the intuitive interpretability of the incoherent image is retained, making it an ideal map with which to position the probe for energy-loss spectroscopy with atomic precision.

As the energy-loss spectrum can be obtained simultaneously with the image (Fig. 1), the probe can be positioned over a single atomic column seen in the image. For an incoherent, large energy-loss scattering event that is also generated close to an atomic core, the *s*-type Bloch states will again be preferentially selected, the probe channelling effect will occur, and a similar description of the resolution in terms of a sharp object function convoluted with the probe intensity profile can be applied. In-

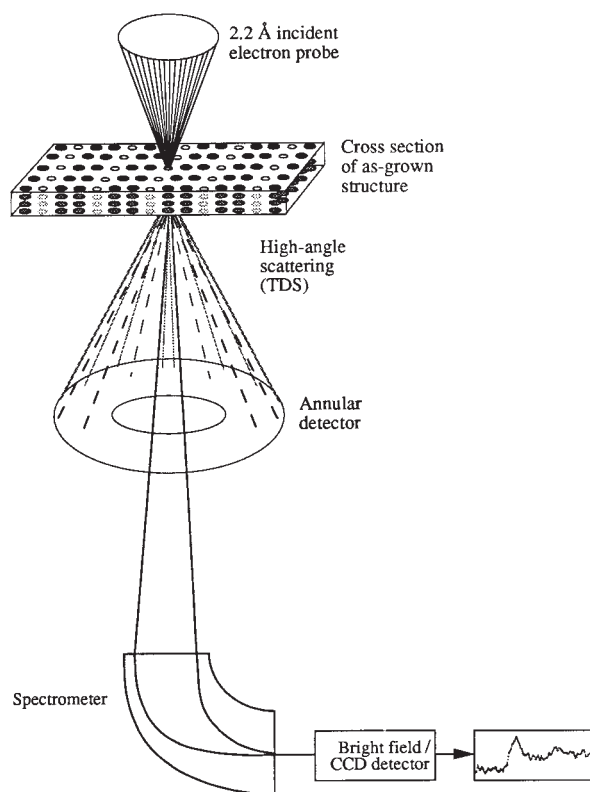


FIG. 1 Schematic diagram of the STEM detectors showing the high-angle scattering used for Z-contrast imaging. Collecting at high angles reduces coherence only to correlations between nearest neighbours of atoms in the same column⁵, and the large angular range of the detector averages any coherent scattering of the outgoing electrons. For thin crystalline materials in major zone-axis orientations, where the probe is smaller than the atomic column separation, a map of the location of the atomic columns can be generated where the intensity is directly dependent on columnar composition.

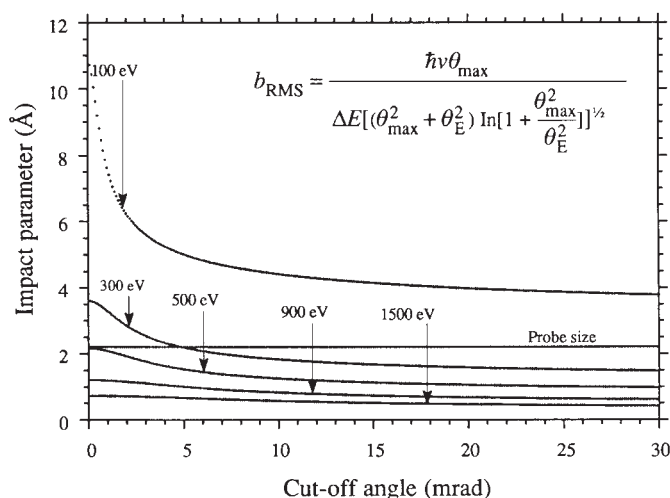


FIG. 2 The range of the inelastic scattering event as a function of the spectrometer collection angle for various energy losses can be estimated from the root mean square impact parameter, b_{RMS} (v is the electron velocity, θ_{max} is the collection angle defined by the aperture size, ΔE is the energy loss and θ_E is the characteristic angle given by $\Delta E/2E_0$, where E_0 is the incident beam energy). Using the maximum collection angle allowed by the inner angle of the dark field detector (30 mrad), for core-losses above 300 eV the impact parameter becomes smaller than the probe, allowing atomic resolution to be obtained^{6,7}. Note that this is a classical free space calculation; dielectric screening may reduce the impact parameter and allow atomic resolution at lower energy losses¹⁶.

elastic signals of this nature would be expected to have the same properties as the Z-contrast image, that is probe channelling will eliminate beam broadening for thicker specimens, allowing the chemical analysis of an individual atomic column within a crystal^{6,7}. To avoid dynamical effects on the outgoing inelastically scattered electrons it is necessary to average over a wide angular range. Under these circumstances, the simple classical description of the impact parameter⁸ approximates well to the full quantum-mechanical results⁹⁻¹¹, and Fig. 2 shows the expected spatial resolution of the energy-loss signal.

This anticipated high spatial resolution is demonstrated by studying epitaxial CoSi₂-Si interfaces produced by high-dose implantation of Co⁺ ions in a heated Si (001) substrate. Annealing results in a continuous buried CoSi₂ layer consisting of {001} terraces separated by occasional {111} facets. Cross-sectional samples, that is with the interface parallel to the beam direction, were prepared for electron microscopy by mechanical polishing and ion-milling. The Z-contrast image of a {111} facet is shown in Fig. 3a, with the brighter region corresponding to the increased scattering power of the heavier cobalt atoms in the silicide. (Note that the silicon atoms in the silicide are unresolved, producing a uniform bright background of similar intensity to that of the silicon columns in the substrate.) The stacking fault visible at the interface means that the separation of the planes across the boundary corresponds to 2.7 Å rather than the 3.1-Å planar spacing of the bulk.

The demagnification required to obtain the 2.2-Å probe used in these experiments reduces the beam current by a factor of 20–30 over conventional STEM conditions. The current density may, however, still be sufficient to cause significant beam damage to the interface. To reduce the dose on any single atomic column, spectra were obtained while the probe was scanned along planes parallel to the interface using an oscilloscope linescan to maintain its position. (The Z-contrast image of the interface was used to select a region where the interface was abrupt for the full width of the scan, indicating no change in spectral intensity moving along the interface would be expected.) To reduce the effects of specimen drift ($\sim 2\text{--}3$ Å min⁻¹) exposure times for the

cobalt $L_{2,3}$ edge were limited to only 5 s (Fig. 3b). Figure 3b shows that using the large aperture to obtain a high degree of localization reduces the energy resolution of the spectra, with the width of the L_3 edge being ~ 5 eV. This resolution, however, is quite sufficient to be able to measure relative changes in intensity across the interface. Two reference spectra were obtained from several planes away from the interface, one in the silicon for background subtraction, and the other in the silicide

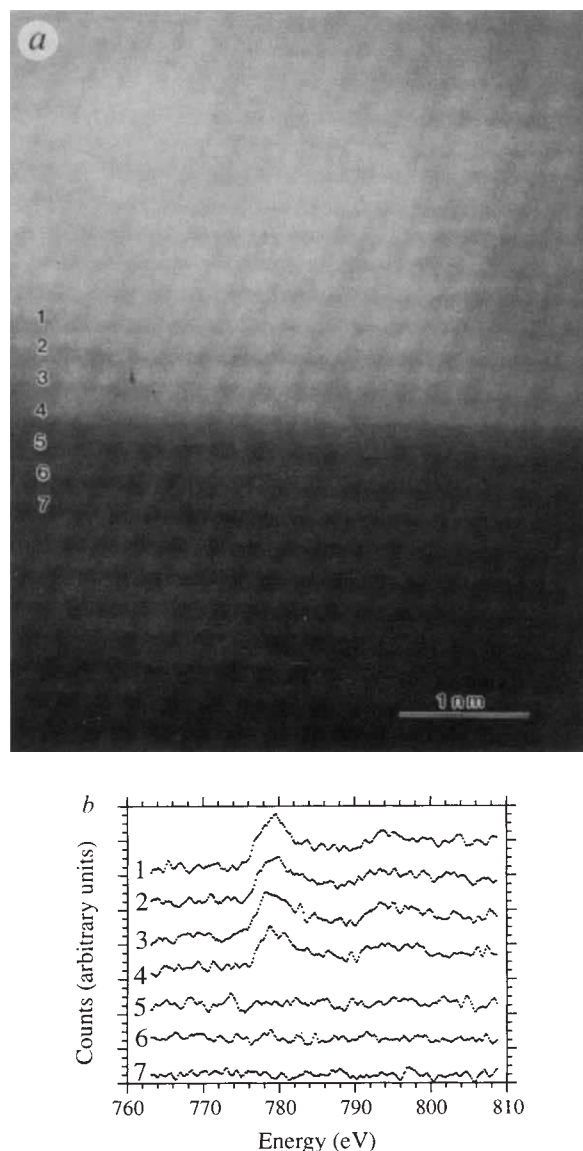


FIG. 3 *a*, Z-contrast image of a {111} facet in a CoSi₂-Si interface, showing the presence of a stacking fault in the silicon which means that the microscopic orientation of the interface is the twinned 'B'-type. Note that phase contrast simulations of such interfaces have not considered a stacking fault¹⁷ resulting in the erroneous interpretation of interfacial cobalt co-ordination¹⁸. *b*, Background-stripped cobalt $L_{2,3}$ spectra obtained plane-by-plane across the interface. The background was subtracted by taking multiple spectra at the same energy loss and exposure time from regions of pure silicon of similar thickness, which were summed to improve statistics. This was then scaled to the background of each spectrum from the interface region. Although the intensity of the background is slightly reduced in the silicide the shape is identical, allowing an accurate subtraction of the background which at the same time removes any variations in detector efficiency. The CCD (charge-coupled device) detector used^{6,7} here has very low thermal and output noise, so that the noise of the spectra is due only to shot noise in the spectrum itself ($\sim 10\%$).

to obtain an accurate measurement of cobalt L-edge intensity. This procedure assumes that the shapes of the spectra are not affected by multiple scattering, an assumption that is borne out by the constancy of the observed $L_3:L_2$ ratio (Fig. 4a). The cobalt profile shows an intensity variation that drops from 86 to 7% in a single atomic plane (Fig. 4b), in good agreement with the image and the predicted spatial resolution. The slight reduction in the contrast at the two planes either side of the interface is most likely due to an amorphous surface layer broadening the probe, or possibly some elastic scattering of the probe out of the channelling condition. It is important to note that for thicker specimens and for elements with higher atomic number, the effect of elastic scattering may become more pronounced.

The linescan in Fig. 4b clearly demonstrates atomic resolution in one dimension. As the Z-contrast image shows two-dimensional resolution, this result indicates that atomic resolution microanalysis from a single column is possible in principle. Col-

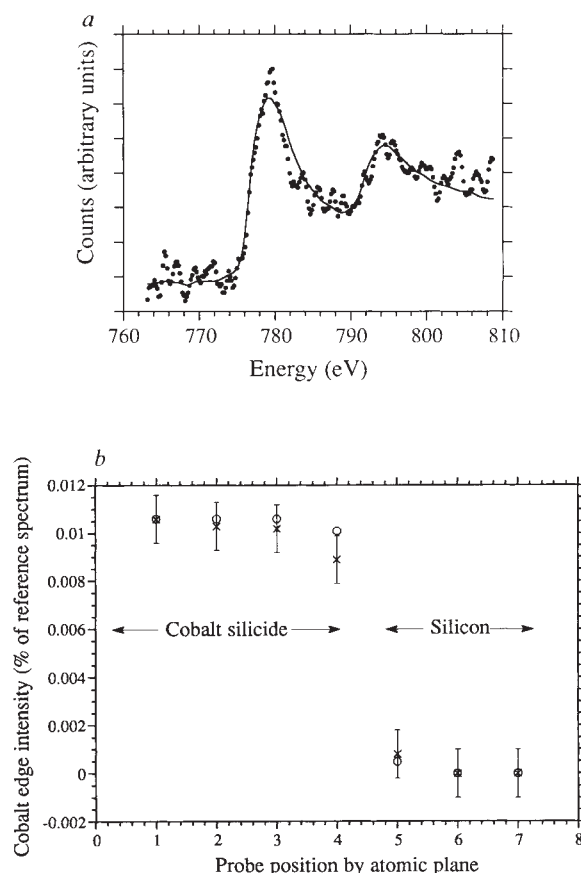


FIG. 4 *a*, The total intensity of the cobalt L-edge from planes close to the interface ($\bullet$) was calculated by fitting a reference spectrum (solid line) obtained from the bulk of the silicide. The reference spectrum was again obtained by summing 20 spectra from areas of similar thickness 4-6 atomic planes from the interface, giving much improved statistics. The reference spectrum was confirmed to be characteristic of bulk CoSi₂ by the characteristic 2:1 $L_3:L_2$ intensity ratio¹⁹. The spectrum was fitted using a least-squares routine for both the pre-edge intensity to set the zero level, and also over a 30-eV window for the total cobalt intensity. (It should be noted that the co-ordination of cobalt at the interface did not appear to vary from that in the bulk, in agreement with recent interface structural models¹⁸, though the signal-to-noise ratio is insufficient to accurately address this point.) *b*, The experimental changes ($\times$) in the L_3 intensity (Fig. 3b) agree well with those expected theoretically from calculations of the probe intensity profile at the surface ($\circ$), and demonstrate clearly that atomic-resolution analysis has been achieved at the interface.

umn-by-column compositional information could be directly related to the interface structure seen in the image. This method overcomes problems caused by beam broadening and uncertainties in the interface structure that occur in microanalysis without the benefit of a high-resolution reference image¹². At an amorphous-crystal interface of course, the usual beam broadening will occur when the probe is in the amorphous layer, but the last atomic column of the crystal can still be analysed with atomic resolution. Additionally, by increasing the number of spectra taken from each plane, it should be possible to interpret the fine

structure of the spectrum in terms of the chemical bonding on the atomic scale¹³, with directional information being obtained through angular deconvolution¹⁴. (Note that in all these situations an increase in the number of spectra is needed rather than a single longer exposure so that the effects of beam damage can be monitored.) How localized the band structure information will be from a single atomic column has yet to be determined, but a knowledge of the exact crystallographic location of the probe greatly reduces some of the ambiguities in the interpretation of spectral fine structure¹⁵. □

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Timing of the Younger Dryas event in East Africa from lake-level changes

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THE last deglaciation was interrupted by an abrupt cooling event, the Younger Dryas, at 11,000–10,000 yr BP (uncalibrated radiocarbon timescale)¹. Originally recognized in climate records from northwest Europe, the Younger Dryas has now been identified in marine and ice-core records worldwide^{2–6}. In the tropics, a broadly contemporaneous change in climate is recorded by decreases in water levels and increased salinity of lakes^{7–9,14}, indicating a period of arid climate caused by a reduction in ocean-to-land moisture flux. The exact timing of these changes in relation to the Younger Dryas event in high-latitude records has remained unclear, however. Here we present climate records based on analyses of diatom assemblages, geochemistry and magnetic mineralogy of radiocarbon-dated sequences of laminated lake sediments from Lake Magadi in the East African rift. These records provide a detailed record of climate change in lowland equatorial Africa throughout the last deglaciation (12,800–10,000 ¹⁴C yr BP). We find that lake-level and humidity maxima coincide with the most rapid phases of ice melting in the Northern Hemisphere, and that the climate changes, including the Younger Dryas event, were synchronous at low and high latitudes. Thus, the effects of abrupt climate change appear to be felt at both high and low latitudes without a significant time lag.

Magadi (1° 50' S, 36° 18' E, 600 m above sea level) is a closed lake at the southern end of the eastern (Gregory) rift (Fig. 1). It is today hypersaline-alkaline, less than 1 m deep with a trona crust, and fed almost entirely by groundwater inflows (modern

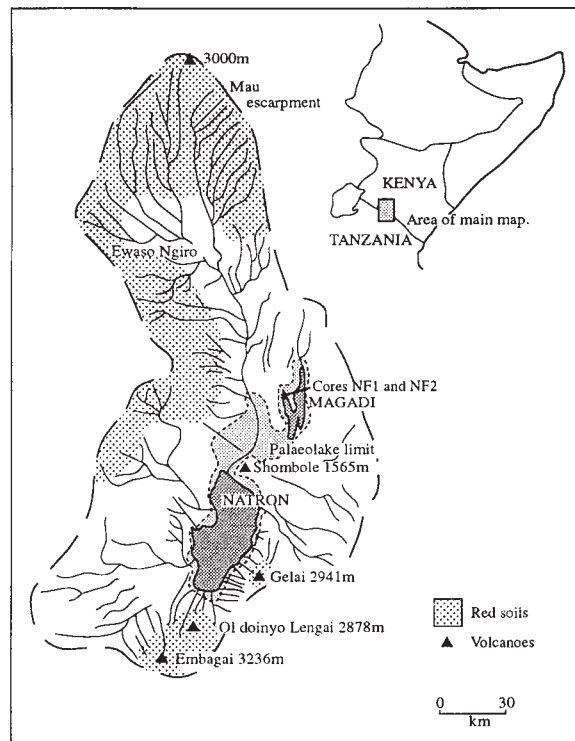
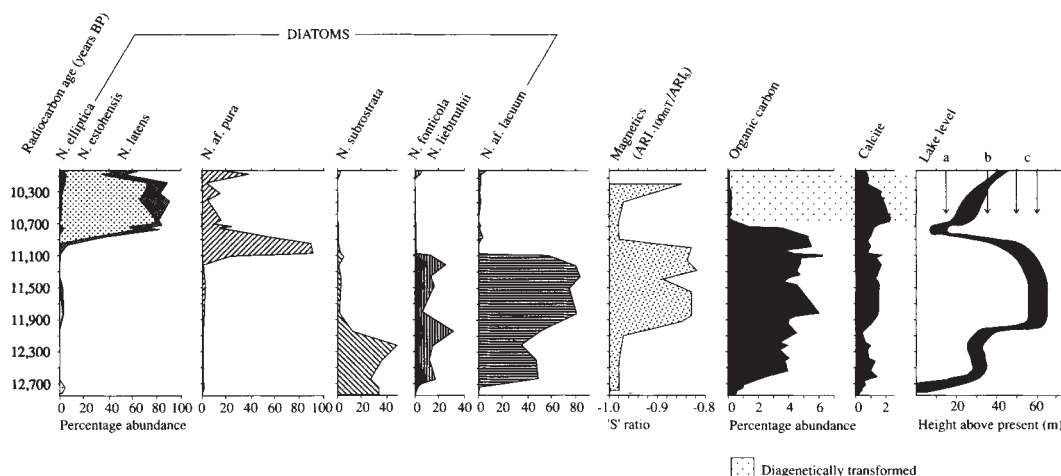


FIG. 1 Location map of the Magadi-Natron catchment, showing the extent of the modern lakes and the combined palaeolake at +60 m elevation.

springs, pH 9.0–9.5, total dissolved solids (TDS) 20–40 g l⁻¹; modern brines, pH ~ 10.5, TDS ~ 300 g l⁻¹)^{10,11}. Like many other East African rift lakes, Magadi contains extensive lacustrine deposits which contain evidence of significant Late Quaternary lake-level changes. These deposits include the High Magadi beds, ¹⁴C dated to the early Holocene (9,120 yr BP), and marginal stromatolites with ¹⁴C and U/Th dates between 12,450 and 9,650 yr BP (ref. 12). The stromatolites form a ring around both

FIG. 2 Diatom, mineral magnetic and geochemical record from the NF cores, Magadi, set against uncalibrated ^{14}C timescale. On the synthesized lake-level curve, 'a' represents the minimum estimated water depth for lake stratification, 'b' is the internal threshold at +35 m separating Lake Natron and Magadi, and 'c' shows the altitude of the stromatolite ring around the combined lake.



Lake Magadi and the adjoining Lake Natron, and were formed by a single water body 50–60 m deep. Unlike Magadi, Natron receives substantial surface water inflows, primarily from the Ewaso Ngiro river (Fig. 1).

Parallel overlapping cores of about 9 m long, were recovered from the Flamingo Nursery (NF) lagoon site in the northwestern sector of Lake Magadi, and these cores have been analysed for mineral magnetics^{13,14}, diatoms^{15,16}, geochemistry and pollen¹⁷, and dated by radiocarbon and U/Th^{18,19}. Between unconformities at 6 cm and 290 cm, the cores contain lake sediments that are continuously laminated, except between 290 and 278 cm, and for a 10-cm silt layer above 110 cm. The dark laminae comprise autochthonous organic matter and diatom frustules, whereas the light laminae are rich in all allochthonous detrital minerals, plus autochthonous carbonates and/or magadiite^{20,21}. Coupled with an absence of benthic diatoms above 278 cm, these laminated sediments testify to the existence of a stratified lake >10 m deep, and suggest lithostratigraphic continuity during the time period of their deposition.

Ten ^{14}C dates obtained by accelerator mass spectroscopy (AMS) on total organic matter provide a chronology for this part of the sequence, with only one minor age reversal and good inter-laboratory agreement (Table 1). Pollen and organic matter are not preserved above 65 cm because of oxidation, and AMS dating has not been possible for this part of the sequence. $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ analyses of modern waters and of Late Pleistocene–Holocene stromatolites indicate that dissolved organic carbon (DIC) in Lake Magadi was in equilibrium with atmospheric CO_2 and that ^{14}C activity levels have not been significantly modified by any 'hard water' effect²². Counting and measurement using X-ray photography and microscope analysis of thin sections indicates that the laminae were not formed annually, and that they have a mean periodicity of 4.3 yr per couplet between the ^{14}C dates at 70 and 232 cm (ref. 20). Current calibration data sets indicate a more or less constant relationship between radio-

carbon and calendar chronologies for the period from 12,000 to 10,700 ^{14}C yr BP (refs. 6, 28). Using our observed empirical relationship between laminae counts and radiocarbon years during this time period, and adjusting ages for the ^{14}C plateau at 10,000 yr BP (ref. 26), the age–depth curve has been extended to include the uppermost part of the sediment for which AMS dates are lacking. The resulting chronology indicates that the upper 290 cm of the NF sequence spans the period from ~12,800 to ~10,000 ^{14}C yr BP.

In Fig. 2, selected multi-proxy palaeohydrological indicators reflecting changes in the Lake Magadi basin are transformed onto a ^{14}C timescale. Diatom assemblages display shifts between different species of *Nitzschia*, primarily reflecting changes in lake-water chemistry, notably pH and conductivity, changes that are also shown by other geochemical indicators. Water-level fluctuations have been inferred from mineral magnetic parameters, such as the reversed field 'S' ratio which relates directly to the presence of haematite. This magnetic mineral is derived from erosion of red ferrallitic soils in the headwaters of the Ewaso Ngiro catchment and was present in Magadi only when it was joined to Lake Natron above the threshold of +35 m (ref. 14). Starting at a low water level, Lake Magadi rose to become stratified by 12,700 yr BP (278 cm), as indicated by the onset of lamination, an abrupt change in diatoms and a more gradual rise in the percentage of organic matter and calcite. By 12,000 yr BP (250 cm), mineral magnetic measures show that Magadi had risen above the +35 m sill to become joined with Natron, and the level of the unified lake continued to rise to reach above the +58 m water level marked by the stromatolite ring. According to diatom transfer functions, the resulting deepwater lake had a pH ~9.4 and was oligosaline¹³. All palaeohydrological indicators are consistent in showing that this lake-level maximum was maintained from ~12,000 until 11,100 ^{14}C yr BP (135 cm).

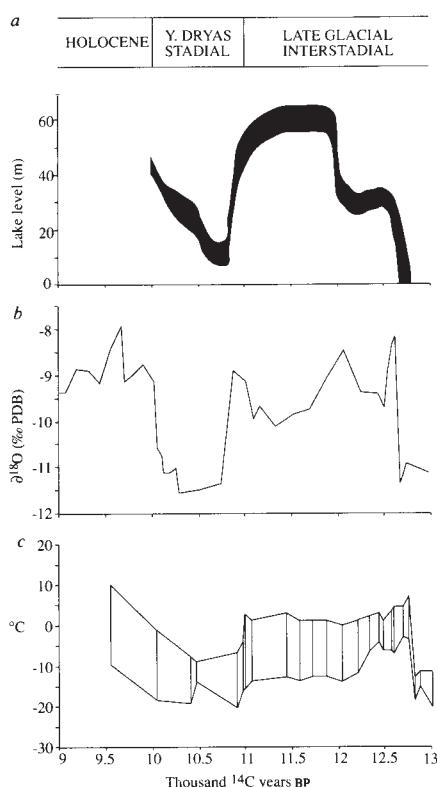
A switch from *N. af. lacuum* to *N. af. pura*, and a fall in the percentage calcite and organic matter at this point marks the beginning of a change in lake-water chemistry to more concentrated and alkaline conditions. The associated fall in lake level led to the separation of Lake Magadi from Natron into two bodies soon after 10,900 yr BP (120 cm), as can be inferred from both the 'S' ratio and diatom assemblages. The lake regression was associated with an increase in the thickness of pale laminae (that is, detrital component) and the water level reached a minimum at 10,800 yr BP when it was shallow enough for lake stratification to break down. From 10,700 yr BP an intermediate-level lake was established, still deep enough to be stratified, but with an increased pH and conductivity (meso- to hyper-saline). Analysis of $^{230}\text{Th}/^{232}\text{Th}$ ratios^{17,23} and of calcite micromorphology²¹ indicate that calcite values after 10,700 yr BP reflect secondary diagenesis rather than syn-depositional conditions. A second transgressive phase is marked towards the top of the NF

TABLE 1 Terminal Pleistocene AMS dates for NF core sequence

	Core depth (cm)	^{14}C age (yr BP)
OxA-3281	70–71	10,700 ± 100
OxA-3282	111–112	10,850 ± 100
Gif-	117–118	10,800 ± 120
OxA-3283	130–131	11,070 ± 100
OxA-3284	139–140	11,110 ± 110
OxA-3285	167–168	11,420 ± 110
OxA-3286	232–233	11,870 ± 120
Gif-	233–234	12,090 ± 120
OxA-3287	269–270	12,840 ± 120
OxA-3288	285–286	12,620 ± 110

Half-life 5,568-yr, corrected for $\delta^{13}\text{C}$ values of about –20 per mil.

FIG. 3 Comparison of records of tropical and temperate palaeoclimate, and of deglaciation, during the last glacial-interglacial transition; a, inferred lake-level curve from Magadi; b, oxygen-isotope curve from Rotsee, Switzerland (from ref. 26); c, reconstructed winter temperatures for Britain from Coleoptera (from ref. 27).



sequence, where a change in magnetics at ~20 cm indicates that the threshold of +35 m was again reached. According to the extrapolated chronology, the second lake-level maximum began at ~10,200 yr BP, although a subsequent hiatus precludes estimation of its duration.

Pollen-derived palaeotemperature estimates for this time period from elsewhere in the East African rifts indicate differences from modern values of <2 °C (ref. 24), sufficient to change potential evaporation at Magadi by <10%. We consequently interpret the reconstructed lake-level curve primarily as a proxy palaeoprecipitation index over the Magadi-Natron catchment. The lake level maxima reflect periods of highest rainfall, interrupted by a drier phase coinciding with the Younger Dryas event. Numerical calibration of this curve is hindered by the complex basin topography and the role of groundwater inflows, but water-balance calculations for the high-level lake (12,000–11,100 yr BP) require a mean increase of 350–450 mm per annum over the catchment for the lake to achieve hydrological equilibrium. This is comparable to, if slightly higher than, palaeoprecipitation estimates from other East African lakes²⁵. The response time of the lake surface area to hydroclimatic change between the equilibrium water level states shown in Fig. 2 is 50–100 yr, assuming no pulsed inputs.

The record of tropical climatic change from Magadi shows a high degree of synchronicity with those for the Late Glacial in Europe (Fig. 3)^{26,27}. Conditions became warmer and wetter around 12,700 ¹⁴C yr BP, cooler and drier at 11,100–10,700 ¹⁴C yr BP, with renewed warming and wetting ~10,000 ¹⁴C yr BP. The effects of radiocarbon age 'plateaux' at 12,800–12,600 and ~10,000 ¹⁴C yr BP makes closer temporal correlation than this difficult to achieve^{6,26}. The similarity of these and other records indicates that this climatic oscillations was not only a global event, but also that there were no significant timelags between tropical and temperate components of the climate system during the last deglaciation. Enhanced moisture flux from oceans to continents at this time was evidently not restricted to the Atlantic sector, and the East African lake record suggest an early reactivation of the Indian Monsoon circulation system at the end of the Pleistocene. □

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Very high rates of bedload sediment transport by ephemeral desert rivers

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GEOMORPHOLOGISTS have thought for some time that rates of sediment transfer might differ markedly in ephemeral and perennial rivers, and have used this idea to explain both the changing character of sedimentary successions and the morphology of rivers in sub-humid or semi-arid areas that have experienced significant shifts in climate during the Quaternary period^{1–5}. But until now there has been a lack of suitable field data to confirm this suggestion, mainly because floods in arid zones are infrequent and unpredictable. Here we present bedload sediment transport data for an ephemeral river in Israel, which show it to be, on average, as much as 400 times more efficient at transporting coarse material than its perennial counterparts in humid zones. This suggests that existing predictive sediment transport equations^{2,4}, developed and calibrated exclusively with data obtained in perennial rivers, are inadequate for application to rivers in arid environments. It also suggests that areas that are at risk of shifting from sub-humid to semi-arid conditions as a result of prospective global changes in climate may suffer severe sedimentation problems.

There is, of course, some evidence of very high loads of suspended sediment that sets ephemeral rivers apart from their perennial counterparts^{6,7}. There are also indications that scour-and-fill of the river bed during the passage of a flood and the consequent release of sediment may be greater in arid zones^{8,9}. Until now, however, there have been no data on downstream flux-rates of bedload (that is sediment that is carried along essentially in contact with the stream bed) in ephemeral channels. Yet bedload has always been thought to make a far greater contribution to sediment yield in desert environments than in those that are more humid.

We have installed sediment monitoring stations on the Nahal Yatir and the Nahal Eshtemoa, two wadis of the Negev Desert near Beer Sheva, Israel. Bedload flux-rates are established automatically and continuously using a number of Birkbeck-type slot samplers that are set into the beds of the rivers^{10,11}, and hydraulic parameters such as bed shear and stream power are determined from continuous records of local water surface slope and stage-discharge relationships. The data we present here arise from the monitoring of five flash floods on the Nahal Yatir. We have contrasted these with the winter 1971 dataset of Oak Creek, Oregon¹² which has provided a basis for much of the controversy that surrounds the nature of bedload transport. This debate centres on whether all clasts are entrained regardless of their mass, or whether hydraulic selection encourages an inverse relationship between net transport velocity and clast size¹³⁻¹⁵.

Figure 1 gives unit bedload flux-rates as a function of specific stream power, ω , which is defined as $\omega = \rho g Y S u$, where ρ is fluid density, g is acceleration due to gravity, Y is water depth, S is longitudinal water surface slope and u is mean flow velocity. The trends in both sets of data are extremely well defined considering what we now expect as normal for sediment transport in gravel-bed channels¹⁶, and the correlation coefficients r are 0.93 and 0.97 for the Yatir and Oak Creek, respectively. The wide separation of the two datasets is remarkable, however. At low values of stream power, transport rates on the Yatir are as much as one million times greater than those on Oak Creek, although this relative difference reduces to one hundred times at the highest measured fluxes. The slopes of the least-squares log-log relationships between bedload flux-rate and specific stream power are 1.22 and 4.46 for the Yatir and Oak Creek, respectively, and a casual extrapolation of these curves suggests that the two streams would behave similarly only if stream power were to approach 300 W m^{-2} .

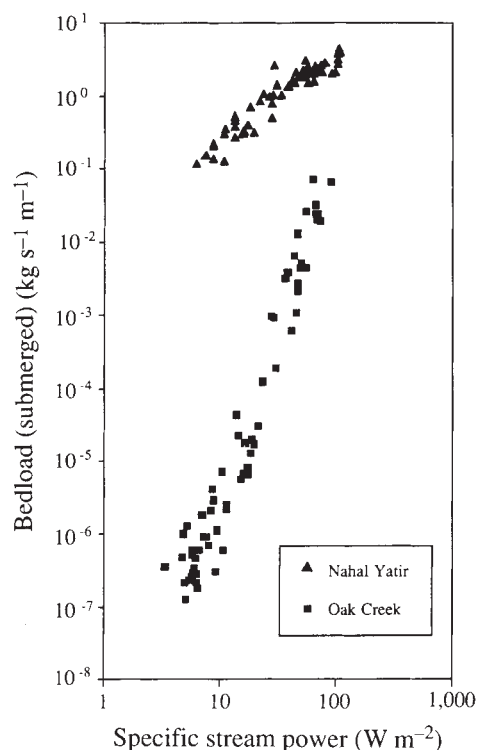


FIG. 1 Unit bedload sediment flux rates (i_b) as a function of specific stream power (ω) for five flash floods on the ephemeral Nahal Yatir, Negev Desert, Israel (this work), and for the winter 1971 dataset¹² of the perennial Oak Creek, Oregon. Both channels have gravel beds. The least-squares regression equations and correlation coefficients (r) are: $\log i_b = -1.90 + 1.22 \log \omega$, $r = 0.93$ for Nahal Yatir; $\log i_b = -9.98 + 4.46 \log \omega$, $r = 0.97$ for Oak Creek.

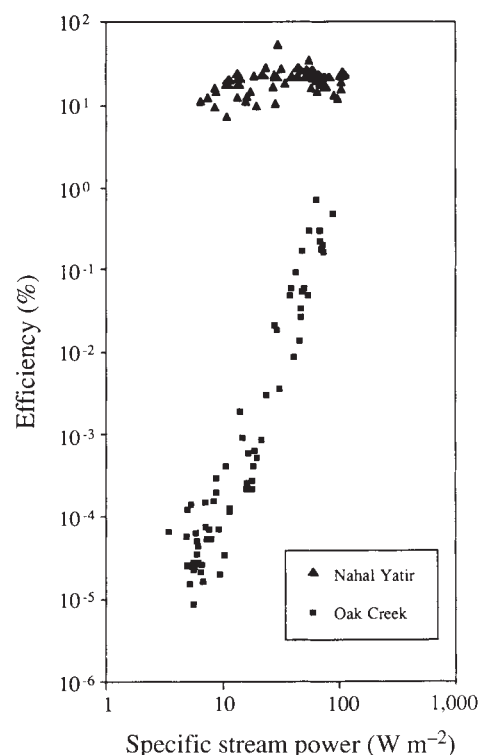


FIG. 2 Efficiency (%) in transporting bedload sediment as a function of contemporary specific stream power for five flash floods on the ephemeral Nahal Yatir, Negev Desert, Israel (this work), and for the winter 1971 dataset¹² of the perennial Oak Creek, Oregon. The correlation coefficients are $r = 0.16$ and 0.74 , respectively. This figure illustrates the effects on sediment availability of the armour layer that commonly develops in perennial channels, and is poorly developed or absent in ephemerals. A shift to ephemeral flows in areas that are now sub-humid might see a drift towards the pattern epitomized by the Yatir and away from that of Oak Creek.

A significant part of the explanation for the differences in bedload transport behaviour lies in the different vertical structure of each stream bed. Whereas both rivers have gravel beds, Oak Creek (like other perennials in humid environments) has a well developed armour layer consisting of coarser clasts and this overlies and protects a finer substrate. In contrast, the Yatir, like other ephemeral or seasonal streams^{17,18} has no armour layer. We have argued elsewhere¹⁹ that poor layer development or non-layering in seasonal and ephemeral streams is a function of the rapid rise and fall in water discharge that is characteristic of flash floods, the high rates of sediment transport and the extended periods of dormancy between floods. These reduce the tendency towards the size-selective transport of clasts and the winnowing of fine material that are each thought to encourage armouring in perennials^{14,20}. In addition, it has been argued by others that sediment supply is a factor that directly governs the degree of armour development²¹, and in arid zones, where hillslope soils are less protected by vegetation, the availability of coarse sediment is much greater. Whatever its cause, poor layer development or non-layering means that ephemeral streams, as represented by the Yatir, are more effective as bulk sediment carriers.

Of course, when assessing the total yield of bedload in ephemeral and perennial streams rather than flux-rates measured over short intervals, the duration and the infrequency of flash floods both have to be taken into account. The records are not long enough for a magnitude-frequency analysis. Nevertheless, a casual comparison of what is essentially the annual yield contained in each of the datasets tends to confirm the Yatir's supremacy despite the fact that it is active for so little time. Per unit

channel width, Oak Creek delivered a bedload only 0.15 times that of Nahal Yatir but in a period 1,350 times longer. Although extreme, these differences in performance are not unusual; Turkey Brook²², another perennial stream but one that has an armour layer that is less pronounced than Oak Creek, delivered one-third of the Yatir equivalent, yet even here, this took 34 times longer to accomplish.

Ephemeral streams are not only more effective as bulk sediment carriers, their performance is also less variable. Figure 2 shows the efficiency of each stream as specific stream power varies. Efficiency (per cent) is defined as $100i_b/(\omega/\tan \alpha)$, where i_b is submerged weight unit bedload flux-rate and α is the internal angle of friction for cohesionless granular material. Not only is the Yatir much more efficient (mean efficiency is 20% against a mean of 0.05% for Oak Creek over approximately the same range of hydraulic conditions), but its efficiency does not vary significantly with stream power (coefficient of correlation $r=0.16$). This contrasts with Oak Creek ($r=0.74$), where direct observation¹² and inference²³ suggest that efficiency is related to the progressive breakup of the armour layer and the exploitation of the finer substrate under high flow conditions.

These marked differences in behaviour lead us to comment, if only briefly, that predictive bedload equations^{15,24} which are calibrated with data from armoured channel-beds such as Oak Creek (the development of which has preoccupied the river sediments community for some time) may require considerable remoulding before they, or their equivalents, are applicable to ephemeral streams. This means that those rivers that have the largest problems of sedimentation (that is those of the world's drylands) are presently unserved for engineering purposes.

The bedload data of the Nahal Yatir provides dynamic confirmation that desert streams behave differently from humid zone counterparts, and that they do indeed deliver greater amounts of coarse sediment as has been speculated from the changing character of sedimentary successions that were laid down during past periods of known climatic fluctuation. We use the contrast between our Yatir data and that of Oak Creek (and other perennial streams not featured here) to speculate that any future shifts in climate may see a transformation of the regional characteristic

flood hydrograph, a change in the sedimentary character of the river channel beds that reduces or eliminates the armour, and a consequent increase in bedload sediment yield in areas that have hitherto been untroubled by significant problems of sedimentation. The rapid loss of water-supply reservoir capacity is one possible upshot, and it may be that the lessons of Tarbella on the Indus, Elephant Butte on the Rio Grande and Lake Mead on the Colorado, which have all suffered from sedimentation, may need to be heeded in those areas that presently enjoy the comparatively low sediment yields characteristic of sub-humid environments. □

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Trade-offs among dispersal strategies in British plants

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TRADE-OFFS are of fundamental importance in population biology. They prevent the evolution of a darwinian demon (a species that breeds fast, lives long and is both a good competitor and disperser) and result in the wide range of life histories we observe in nature¹. Trade-offs are also thought to be important in ecology, driving successional change and maintaining species diversity^{2,3}. But the demonstration of trade-offs is difficult^{4,5}. Evolutionary theory predicts trade-offs between seed dormancy and (1) seed weight⁶ and (2) seed spatial dispersal^{6,7}. Here we present simulation results predicting a third trade-off between seed dormancy and adult longevity. The existence of these trade-offs is tested, to our knowledge for the first time, using modern comparative methods^{8,9} and data from long-term experiments^{10–12}. The analyses are consistent with the existence of all three trade-offs in nature.

All environments vary and organisms exploiting them are likely to evolve adaptations for dealing with fluctuations in favourability. Life-history theory has shown that seed dormancy, dispersal and iteroparity may evolve in response to environ-

mental uncertainty^{6,7,13–15}. Consider a simple environment where the conditions are suitable for growth and reproduction in some years but in other years reproduction fails completely. In such an environment an annual plant genotype with no seed dormancy would maximize its arithmetic average population growth rate but become extinct the first time reproduction failed. A genotype that never germinated would also become extinct as a result of seed mortality. Hence in a variable environment an intermediate germination strategy is optimal¹³. Moreover, theory predicts that life-history attributes that reduce the impact of environmental variation will show patterns of negative covariation^{6,7,14}. For example, species with efficient seed dispersal reduce the likelihood that all seeds will be exposed to unfavourable conditions in any one year, and so we would expect a negative relationship between the efficiency of seed dispersal and dormancy. It has also been suggested that a trade-off between spatial and temporal seed dispersal might arise from physical and biochemical constraints¹⁶ or sibling competition¹⁷. Likewise, species with large individual seeds are predicted to have reduced dormancy because their seedlings can draw on a larger food reserve, and hence establish in relatively unfavourable environments. This reduces the realized variance in environmental quality, which selects against seed dormancy⁶.

There are no theoretical studies indicating how dormancy will evolve in response to adult longevity. Some argue that annual plants occur in less 'stable' environments and this selects for more dormancy in annuals than perennials¹⁸. But this argument

has been dismissed by others because there is no reason why, from a seed's point of view, deserts, early successional and other 'unstable' habitats are any more unpredictable for seedling establishment than later successional 'stable' habitats¹⁹. It has also been argued that perennial species have larger seeds which suffer higher rates of loss to herbivores, and this, in turn, selects against dormancy²⁰. Note, however, that when perennials do have larger seeds then, according to the argument described above, they should also have less dormancy. Late-successional trees often have saplings that can survive for extended periods in deep shade; this represents an alternative strategy to the formation of a seed bank²¹. The converse of the arguments presented, that long-lived species should have more dormancy, has also been suggested²². Simulation studies (Table 1) demon-

TABLE 1 Effect of adult longevity and seed survival probability on ESS germination probability

Average adult longevity (years)	1	2	5	10	100
Variation in seedling survival ($P_{ss}=0.5$, constant adult fecundity ($P=1$))					
$P_s=0.9$	0.27	0.37	0.91	1.0	1.0
$P_s=0.1$	0.49	1.0	1.0	1.0	1.0
Variation in adult fecundity ($P=0.5$), constant seedling survival ($P_{ss}=1$)					
$P_s=0.9$	0.27	0.29	0.30	0.31	0.32
$P_s=0.1$	0.49	0.49	0.49	0.49	0.49
Variation in both adult fecundity and seedling survival ($P_{ss}=P=0.5$)					
$P_s=0.9$	0.15	0.23	0.29	0.31	0.32
$P_s=0.1$	0.25	0.49	0.49	0.49	0.49

The evolutionarily stable strategies (ESSs) were determined for a simple stage-structure model which followed both the number of established adults, N , and the number of seeds, S . The model assumes that P_a adults survive from one year to the next. In those sites where adults have died, recruitment occurs as a result of a fair lottery between seedlings. It is assumed that both seedling establishment and adult fecundity are random variables varying from year to year. A population model for the j^{th} genotype incorporating these assumptions has the following form,

$$S_{t+1,j} = P_s(1 - g_j)S_{t,j} + FX \left\{ Y \left(K - P_a \sum_i N_{t,i} \right) \left(g_j S_{t,j} / \sum_i g_i S_{t,i} \right) + P_a N_{t,j} \right\}$$

$$N_{t+1,j} = P_a N_{t,j} + Y \left(K - P_a \sum_i N_{t,i} \right) \left(g_j S_{t,j} / \sum_i g_i S_{t,i} \right)$$

where P_s is the probability of seed survival, g_j is the germination probability of the j^{th} genotype, K is the number of microsites, F is the per capita fecundity and X , Y are binary random variables taking the values 0 and 1. P is the probability of successful reproduction ($X=1$) and P_{ss} the probability of successful seedling establishment ($Y=1$). The seed equation has two parts: the first represents the seed bank, and the second represents the current seed production from new recruits and established plants. The established plant equation also has two components: the first is adult survival, and the second is recruitment into unoccupied microsites. The ESSs were determined by iterating the equations for two genotypes and determining the germination fraction that eliminated all others by a systematic search of the unit interval. Evolutionary stability of the putative ESS was checked by comparison with published results⁷, available for annuals only, and by the numerical computation of the dominant Lyapunov exponent²⁹. Three types of variability are considered: (1) variation in seedling survival, (2) variation in adult fecundity and (3) variation in both adult fecundity and seed survival. In all cases providing the probability of seed survival was not too small the ESS germination probability increased as adults became longer-lived. The models are designed to allow the direction of selection on seed germination to be determined as adult plants become long lived. The models assume that current seed production is an equally efficient mechanism of exploiting a disturbance as having a seed bank. This is often true, however, a seed bank allows disturbances to be exploited rapidly whereas current seed production may involve a delay of several months. In general this will tend to select for increased seed dormancy. The models are easily extended to include such complications and these results will be presented elsewhere³⁰.

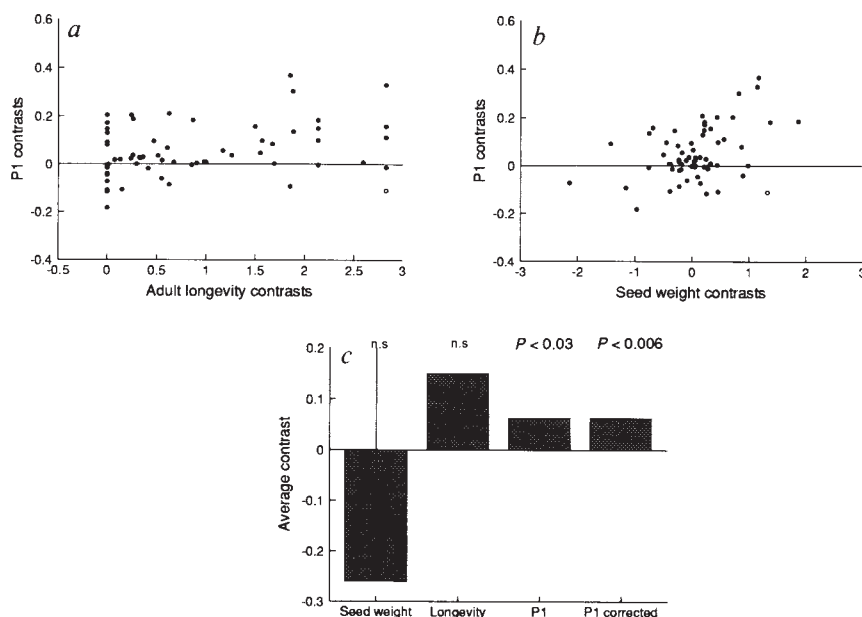
strate that increased adult longevity can select strongly against the evolution of dormancy even if annuals and perennials only differ in their longevity and both experience the same pattern of environmental variability. The effect is greatest when seed survival is high, there is little variation in adult fecundity, and a variable probability of successful seedling establishment. Under these conditions, seed production is effectively de-coupled from seedling establishment, neutralizing the effect of variation in seedling establishment, and thus selecting against dormancy. This pattern of variation is highly plausible because seedlings are extremely sensitive to a wide range of biotic and abiotic factors²³. Note, however, that when adult fecundity is variable, the models predict that even long-lived perennial species should exhibit substantial seed dormancy. Thus, long-lived species characteristic of ecosystems that are regularly burnt should possess substantial dormancy, as is the case²⁴.

Hence we would expect seed dormancy to be negatively correlated with adult longevity, seed size and the efficiency of spatial dispersal. These theoretical predictions were tested using the method of independent contrasts^{8,9}. Data on seed dormancy for 171 species were obtained from experimental studies using a standard protocol, where the pattern of seedling emergence, from single species, even-aged populations was monitored over a 5-year period¹⁰⁻¹². The fraction of all seedlings observed that emerged in the first year of the experiment, termed P_1 , was used as a measure of seed dormancy. Species with strongly dormant seeds will have low values of P_1 as few seedlings emerge early in the experiments, whereas those with little dormancy will have values near unity. Note that because seed mortality is unobserved, we cannot interpret P_1 as the probability of seed germination²⁵. Information on plant life histories and taxonomy was obtained from standard sources^{26,27}. The species represent 34 families and 114 genera, covering a wide range of life histories from annual herbs to perennial grasses. There were no tree species in the sample. The analyses (Fig. 1) demonstrate that species with long-lived adults do have less dormancy, as predicted, even after correcting for the effects of seed size (Fig. 1a). This reduction in seed dormancy with increasing adult longevity seems to be widespread in temperate plant communities: for the Sheffield flora²⁶, 77% of annual species have a persistent seed bank compared to 47% of perennials, and for eastern USA herbaceous plants²⁸, 45% of annuals have full seed dormancy compared to 18% of perennials. Large-seeded species also have less dormancy than small seeded ones; again the effect was highly significant even after correcting for adult longevity (Fig. 1b). There was no significant effect of adult longevity on seed weight (regression slope = 0.11, $P=0.096$) or of seed weight or adult longevity on the likelihood that a species would have efficient spatial dispersal. But after correcting for seed size and adult longevity, there was a highly significant reduction in seed dormancy in those species that had efficient means of spatial dispersal (Fig. 1c).

All comparative analyses are open to alternative interpretations. The trade-offs demonstrated might arise as a result of covariation of the measured life history traits with other unmeasured life history or environmental variables. For example, annuals and perennials typically occur in different habitats. So if the probability of seed survival was lower in habitats containing mainly perennial species than in those with mainly annuals, then this could produce a spurious relationship between adult longevity and dormancy, because the cost of forming a seed bank was higher for perennials. But seed mortality is linked with seed size²⁰ and there is a significant effect of adult longevity even after correcting for seed size. Unfortunately, because the species used in the analyses came from a wide range of habitats, the annuals from 18 and the perennials from 23, classifying the data by habitat produced no meaningful comparisons because of small sample sizes.

The trade-offs demonstrated here involve ecologically important characters that directly influence plant community structure.

FIG. 1 Comparative analysis of the effect of life history traits on the evolution of seed dormancy. Independent contrast methods have been used in all analyses^{8,9}. These methods use phylogenetic or taxonomic information to construct independent contrasts which represent weighted differences between taxa. Technical details describing the construction and analysis of independent contrasts are given in detail in ref. 8. When calculating contrasts for continuous variables (such as P1, adult longevity and seed weight), the taxa with the higher value for adult longevity was used as the reference taxa; as a result, all the contrasts for adult longevity are positive. The contrasts for the other variables can be either positive or negative. Note that analyses using either P1 or seed weight to determine the reference taxa produced similar results. Hence if we are looking at the relationship between adult longevity and P1 and we find that large longevity contrasts generally have positive P1 contrasts, this would mean that as species become longer-lived the value of P1 increased. Therefore, increases in adult longevity are associated with reductions in seed dormancy. Likewise, if P1 contrasts increase as seed weight contrasts increase, this demonstrates that large seeded species generally have higher values of P1, indicating that they have less seed dormancy. *a*, There is a highly significant positive relationship between adult longevity and P1 (slope of regression = 0.045, $P=0.00045$), indicating that species that are longer-lived have less seed dormancy. Adult longevity was determined using a simple scoring system: annual, 1; biennial, 2; monocarpic, 3; perennial, 5. Species in composite categories were given a weighted score: the first term given a weight of 1, the second 0.5 (for example, annual/perennial = $(1 + 0.5 \times 5)/(1 + 0.5)$). *b*, The relationship between log seed weight and P1 is also positive and highly significant (slope of regression, 0.075; $P=0.00069$), indicating that larger seeded species have less seed dormancy. In a multiple regression analysis, where both adult longevity and seed weight were included in the model, both regression slopes were highly significant (longevity $P=0.00021$, seed weight $P=0.0034$, $r^2=0.31$). Regression diagnostics for the multiple regression model identified a single influential, outlying data point (open circle). Exclusion of this data point dramatically increased the significance of both adult longevity and seed weight (multiple regression, longevity $P=0.000006$, seed weight $P=0.0002$, $r^2=0.42$). This outlying point is a comparison of *Vicia hirsuta* and *Vicia cracca*. One possible explanation of this outlying comparison is that the differences in seed weight result largely from variation in the thickness of the seed coat and do not reflect variation in food reserves. A thick seed coat must be broken down before germination can occur, which results in the larger seeded species being more not less dormant. Spatial seed dispersal was ana-



lysed as a discrete variable: species either have efficient seed dispersal or they do not. In all analyses that included seed dispersal, it is always used as the reference variable when calculating contrasts. This allows the maximum number of informative contrasts between taxa that have and do not have efficient spatial dispersal to be constructed. *c*, The effect of efficient seed dispersal on seed weight, longevity and P1. Seed dispersal was assessed using the classification in ref. 26; species with seeds unspecialized for dispersal, or dispersed by ants, or shed from a capsule, were assumed to have inefficient spatial dispersal, whereas species with seeds having adhesive burs, awns, spiny calyx, wings, plumes, adhesive mucilage, or those dispersed as a result of ingestion by animals or dispersed aquatically, were assumed to have efficient spatial dispersal. A positive average contrast indicates that the presence of efficient dispersal mechanisms is associated with an increase in the character considered; a negative average contrast indicates the converse⁹. The P1 corrected bar refers to the association between seed dispersal and dormancy, having first corrected for the effects of seed size and adult longevity on dormancy. The significance levels are from 1-tailed t-tests; the vertical lines are a single standard error directed towards the x-axis. Changes in seed weight and adult longevity did not affect the likelihood that a species would possess an efficient seed dispersal strategy. But species with less seed dormancy (high values of P1) were significantly more likely to possess efficient seed dispersal strategies. This result became more significant after correcting for adult longevity and seed weight.

Understanding how evolutionary forces determine the nature of such trade-offs will undoubtedly lead to a deeper understanding of community structure. □

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Episodic multiregional cortical coherence at multiple frequencies during visual task performance

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THE way in which the brain integrates fragmentary neural events at multiple locations to produce unified perceptual experience and behaviour is called the binding problem^{1,2}. Binding has been proposed to involve correlated activity at different cortical sites during perceptuomotor behaviour³⁻⁵, particularly by synchronization of narrow-band oscillations in the γ -frequency range (30–80 Hz)^{6,7}. In the rabbit olfactory system, inhalation induces increased γ -correlation between sites in olfactory bulb and cortex⁸. In the cat visual system, coherent visual stimuli increase γ -correlation between sites in both the same and different visual cortical areas⁹⁻¹². In monkeys, some groups have found that γ -oscillations transiently synchronize within striate cortex¹³, superior temporal sulcus¹⁴ and somatosensorimotor cortex^{15,16}. Others have reported that visual stimuli produce increased broad-band power, but not γ -oscillations, in several visual cortical areas^{17,18}. But the absence of narrow-band oscillations in itself does not disprove inter-regional synchronization, which may be a broad-band phenomenon. We now describe episodes of increased broad-band coherence among local field potentials from sensory, motor and higher-order cortical sites of macaque monkeys performing a visual discrimination task. Widely distributed sites become coherent without involving other intervening sites. Spatially selective multiregional cortical binding, in the form of broad-band synchronization, may thus play a role in primate perceptuomotor behaviour.

We recorded local field potentials (LFPs) from up to 15 cortical sites in one hemisphere of adult rhesus macaque monkeys (Fig. 1) performing a visual pattern discrimination task and found task-related multiregional synchronization over the entire frequency range examined. Our results are compatible with the hypothesis that perceptuomotor integration involves the broad-band binding of widespread cortical sites.

A total of 5,827 correctly performed trials from three monkeys (G.E., T.I. and L.U.) were studied, including 2,974 trials with a response (GO) to one visual pattern type and 2,853 trials with the response withheld (NO-GO) to another type. The trans-cortical field potential at each site was localized by differential recording with a surface-to-depth bipolar electrode. The amplifier reduced common contributions to the two electrode tips by more than 10,000 times, eliminating propagated fields from sources more than a few millimetres away. The precision of localization was demonstrated in LFP averages which, following the visual stimulus, showed visual receptive field specificity in striate and prestriate regions but not in others. All LFP averages had a high degree of day-to-day reliability over multiple recording sessions covering weeks to months.

Coherence and phase spectra, the frequency domain equivalent of temporal cross-correlation, were used to test for inter-site synchronization over a range of frequencies¹⁹. Multiple episodes of elevated coherence, lasting 50–200 ms and involving multiple frequencies, were observed after the stimulus (Fig. 2). Changes in coherence over time generally followed the same pattern at multiple frequencies. The range of peak times for these

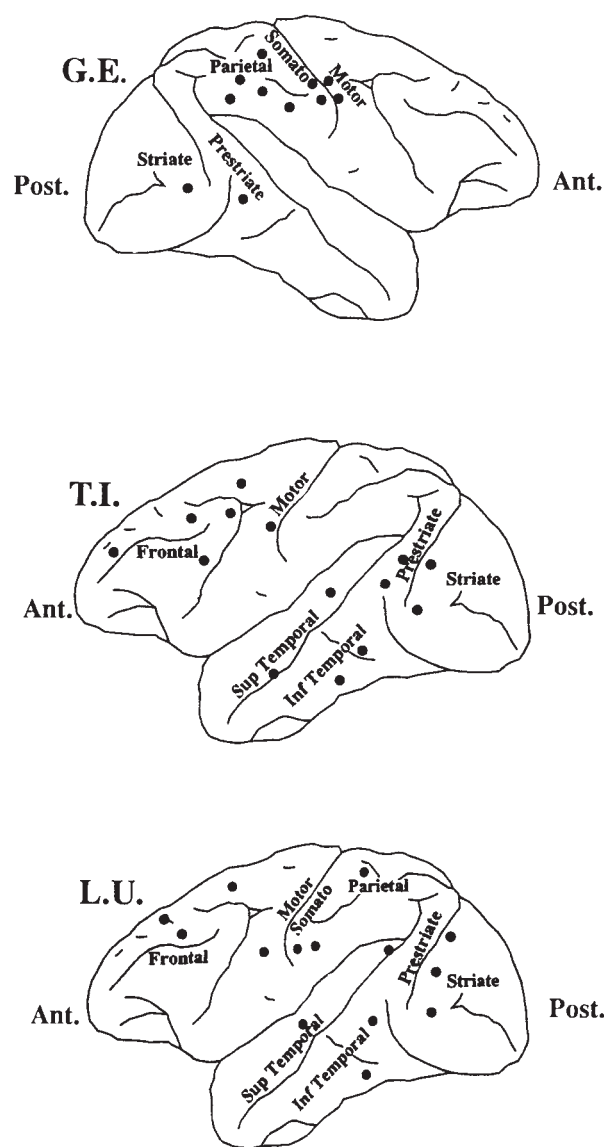
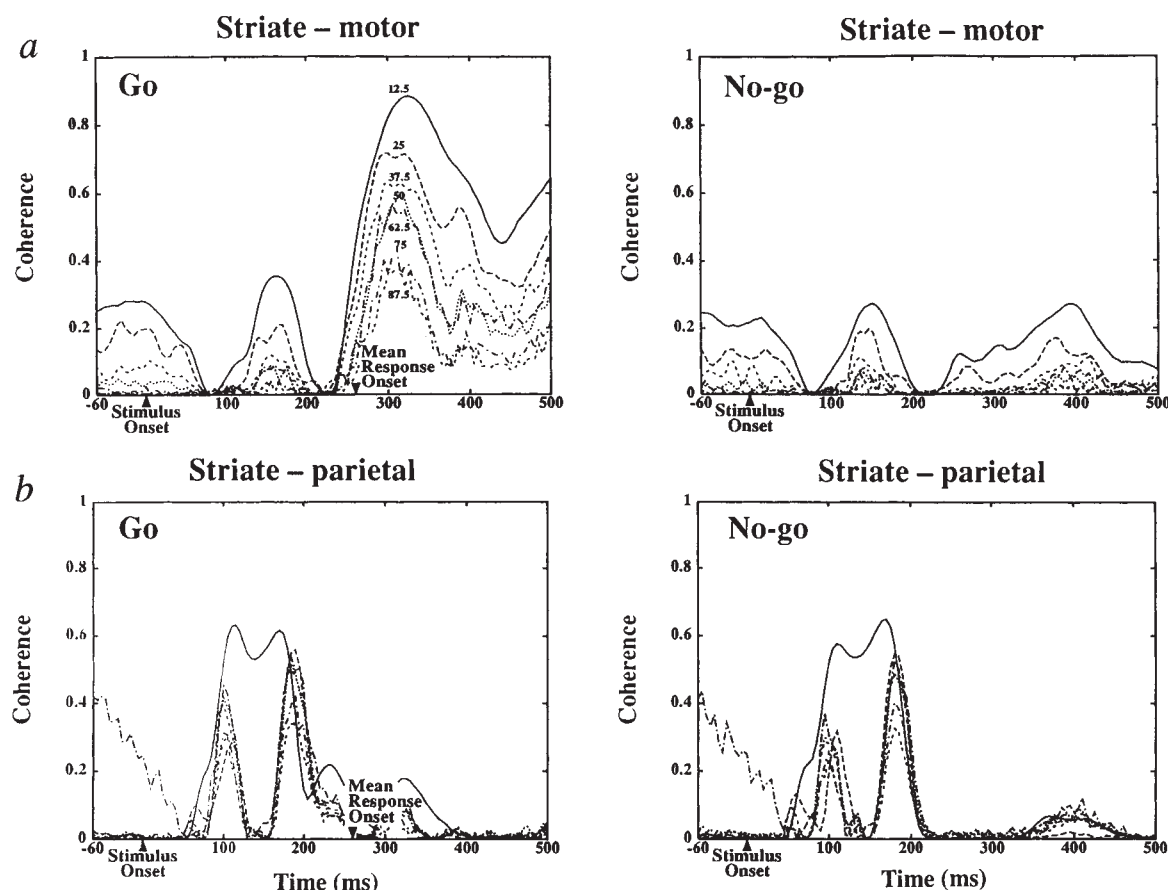


FIG. 1 Locations of recording sites in monkeys G.E., T.I. and L.U. At each site, the signal from a transcortical bipolar electrode (51- μ m-diameter Teflon-coated platinum wires: 2.5 mm tip separation, 0.5 mm exposed surface per tip, less-advanced tip at the pial surface) was differentially amplified by a Grass model P511J amplifier (-6 dB at 1 and 100 Hz, 6 dB per octave falloff), digitized at 200 samples s^{-1} , and stored in 12-bit digital form.

METHODS. Experiments were done at the Laboratory of Neuropsychology at NIMH. Animal care was in accordance with institutional guidelines. Surgical procedures were as described in ref. 26. Monkeys were trained to accept chair restraint readily, to respond (GO condition) to one visual pattern type (line or diamond), and to withhold responding (NO-GO condition) to the other. Equiprobable GO and NO-GO trials were randomly presented in 1,000-trial sessions lasting about 45 min (inter-trial interval randomly varied between 0.5 and 1.25 s). The monkey initiated each trial by depressing a lever with the preferred hand. The head was held 57 cm from the display screen, with 1 deg cm^{-1} visual angle. The computer-generated visual stimulus was presented for 100 ms through a silenced piezoelectric shutter, beginning about 115 ms after data collection began. On GO trials, a water reward was provided 500 ms after stimulus onset if the hand was lifted within 500 ms. On NO-GO trials, the lever was depressed for 500 ms. The stimulus set consisted of four diagonal patterns with two dots (9 mm per side) at opposite corners of an outer square, 6 cm per side, and two dots at opposite corners of a concentric inner square, 2 cm per side. The outer and inner dots were slanted in the same direction in two patterns (lines), and in opposite directions in the other two (diamonds). Thus, no single dot could be used to discriminate between lines and diamonds.

FIG. 2 Coherence time-series for two different site pairs in monkey G.E. Time-series for 7 frequency bins (12.5, 25, 37.5, 50, 62.5, 75 and 87.5 Hz) are displayed in each plot. Left, GO condition (488 trials). Right, NO-GO condition (482 trials). *a*, There is a large increase in broadband coherence between striate and motor sites at the time of the response in the GO condition. *b*, The same striate site shows elevated broadband coherence with a parietal site between 100 and 200 ms post-stimulus. (The striate site does not show significant coherence with other intervening sites.)



METHODS. Spanning the period from 115 ms before until 500 ms after stimulus onset, coherence and phase spectra were computed at each of a series of 80-ms time windows. The spectra had frequency bins at 12.5 Hz (1 cycle per 0.08 s) intervals. Coherence time-series, formed for each frequency bin as the time window was stepped, point by point, across the analysis period, were computed for all pairwise combinations of sites for both GO and NO-GO conditions in each session. All GO or NO-GO trials from a session were used to estimate each site pair's coherence. For statistical evaluation, the Fisher z-transform was first applied to the bounded coherence distribution to yield a roughly normal one. Six sessions were examined: two from each of the

three monkeys. Coherence values were considered significant when exceeding the 95% confidence limit for the mean coherence over all site pairs from both conditions in the analysis period of a session. Two-way analysis of variance was done for each site pair (response condition and time as major effects, frequency bins as cases). Significance of between-condition difference was determined at the 95% level after correction for multiple comparisons by the Bonferroni method. Scheffé comparisons were later made to determine the time points showing significant between-condition differences.

episodes extended from 90 ms after stimulus onset to beyond response onset.

Coherence was significantly above baseline in at least one frequency bin during the analysis period for about 66% of the site pairs in G.E., 93% in T.I. and 90% in L.U. Data became significant in all bins by about 37% of the site pairs in G.E., 56% in T.I. and 20% in L.U. The sites involved were in striate, prestriate, inferotemporal, superotemporal, posterior parietal, somatosensory, motor and frontal cortices. In no case were two sites coherent with zero phase variance over trials, indicating that synchronization was not simply an externally imposed artefact.

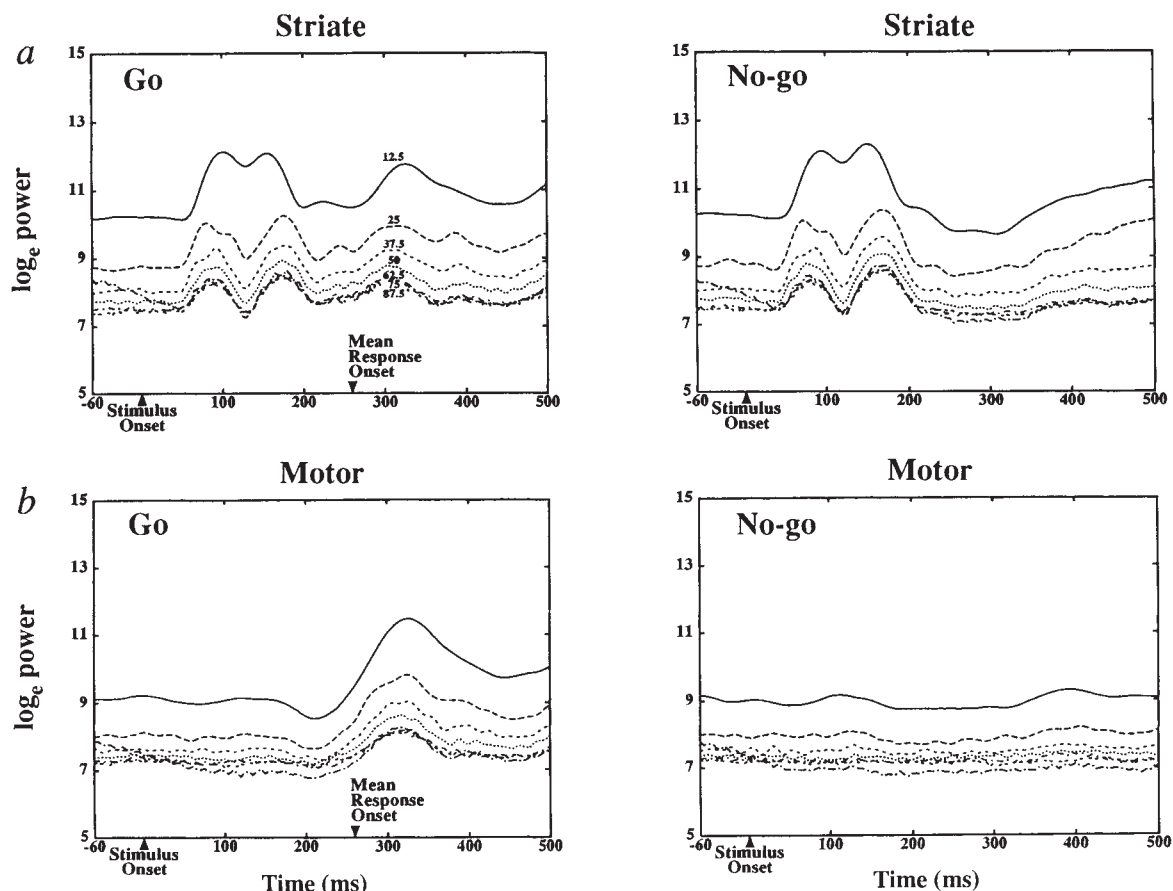
Of the site pairs having significant coherence in all bins, 50% had a significant difference between coherence in the GO and NO-GO conditions. The mean time of significant between-condition difference (285 ± 24 ms) occurred shortly after the mean response onset time (279 ± 15 ms). Thus, between-condition differences in coherence (Fig. 2a) appeared to be largely related to response processing. Signal power (Fig. 3) also significantly differed between conditions at 60% of the sites (mean time of significant difference = 319 ± 20 ms). Power was significantly different for at least one site in 96% of those site pairs having significant between-condition coherence differences, and for both sites in 54% of the pairs. Like coherence, changes in power were generally similar across frequencies, with no evidence of a concomitant low-frequency decrease and γ -frequency increase as has been previously suggested^{6,20,21}.

Single trials were identified in which the LFP waveforms from two sites were synchronized during the time when their coherence was elevated (Fig. 4). Like coherence overall, cross-correlation on these trials was also elevated because of waveform synchronization (Fig. 4a). Synchronization of γ -frequency components was not apparent because of their relatively small amplitude. But when γ -frequency waveforms were isolated by digital filtering, their synchronization was also evident (Fig. 4b).

Elevated coherence, rather than simply appearing because of common activation of multiple cortical areas by an imposed stimulus, manifested spatially and temporally complex patterns in the period after stimulus presentation, when the monkeys had to discriminate successfully between two visual forms and then perform correctly. These task-related patterns of multiregional synchronization are consistent with those previously observed below 10 Hz in human scalp-recorded averaged event-related potentials²²⁻²⁴. But the finding of broadband elevated coherence during the motor response differs from those of studies in monkey^{15,16} and cat²⁵ reporting narrow-band γ -synchronization in relation to motor activity.

The rapid rise in coherence over a broad frequency range during specific stages of task processing may reflect instantaneous inter-regional cortical coupling on multiple timescales, providing a flexibility to cortical information processing not available through operation on a single characteristic timescale. The present results suggest that synchronization can potentially

FIG. 3 Average power time-series for the striate and motor sites of Fig. 2a. Time-series for 7 frequency bins (12.5, 25, 37.5, 50, 62.5, 75 and 87.5 Hz) are displayed in each plot. Power was down nearly to the size of a single amplitude step at 100 Hz. The striate site (a), but not the motor site (b), shows elevated power after stimulus onset. Both the striate and motor sites show elevated power after response onset in the GO condition (left), but not at this time in the NO-GO condition (right). Averages were over 488 GO and 482 NO-GO trials. METHODS. Spanning the period from 115 ms before until 500 ms after stimulus onset, power spectra were computed by the Fast Fourier Transform for the same series of 80-ms time windows used in computing coherence. The power spectra also had frequency bins at 12.5 Hz intervals. Power time-series were computed for



all sites for both GO and NO-GO conditions and averaged over the trials of each session. Similar statistical procedures were used in determining significance as for coherence.

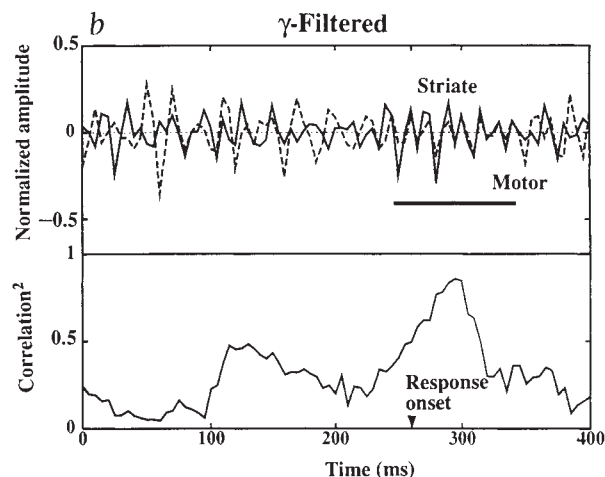
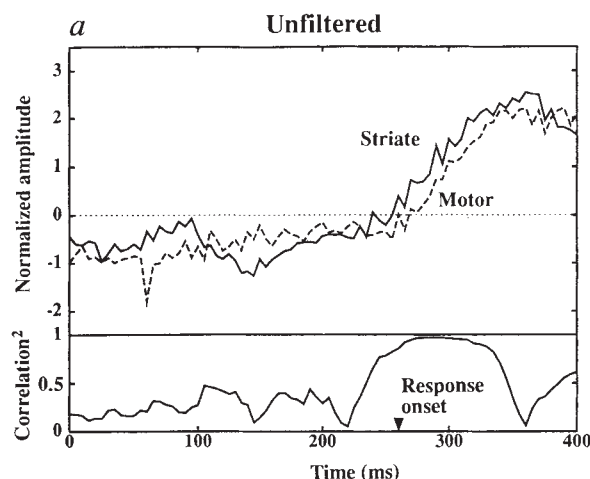


FIG. 4 a, Unfiltered waveforms from the striate and motor sites of Fig. 2a for a single GO trial, with their squared cross-correlation plotted below as a function of time. The period of elevated cross-correlation in this trial corresponds to the broad-band peak in coherence (over all GO trials) in Fig. 2a. b, The waveforms in a after digital filtering to show activity in the γ -frequency range. The period of synchronization (marked by the bar), corresponding to the cross-correlation peak below, demonstrates that the similarity in single-trial temporal structure extends to the γ -range. The maximum squared cross-correlation value (0.86 at

295 ms) exceeded the upper 95% confidence limit (0.50) for the mean value of the time-series.

METHODS. Cross-correlation functions were computed on single-trial records for the same 80-ms time windows used to compute coherence. Cross-correlation time-series were then constructed using the maximum of the squared cross-correlation function in each window. The γ -frequency signals were obtained by time-domain convolution with a bandpass (-6 dB at 30 and 80 Hz, 864 dB per octave falloff) digital filter.

occur between any cortical areas, and provide direct evidence in support of theories^{1,2} proposing that multiregional binding underlies whole-brain functions such as perception and action. □

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Different types of calcium channels mediate central synaptic transmission

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SYNAPTIC transmission is mediated by calcium entry through voltage-dependent calcium channels in presynaptic nerve terminals^{1,2}. Various types of calcium channel have been characterized in neuronal somata^{3–6}, but it is not clear which subtypes induce transmitter release at central synapses. The N-type Ca^{2+} channel blocker ω -conotoxin GVIA (ω -CgTx) suppresses the excitatory postsynaptic responses only partially^{7,8}, whereas potassium-induced release of glutamate from brain synaptosomes can be blocked by ω -Aga-IVA (ref. 9), a blocker of P-type calcium channels^{5,10} and possibly of other types of calcium channels^{11,12}. Here we test type-specific calcium-channel blockers on postsynaptic currents recorded from neurons in thin slices of rat central nervous system¹³. Inhibitory postsynaptic currents in cerebellar and spinal neurons and excitatory postsynaptic currents in hippocampal neurons are markedly suppressed by ω -Aga-IVA and reduced to a lesser extent by ω -CgTx. The L-type calcium channel blocker nifedipine had no effect. Our results indicate that at least two types of calcium channel mediate synaptic transmission in the mammalian central nervous system.

Inhibitory postsynaptic current (i.p.s.cs) were evoked in neurons of the deep cerebellar nuclei by stimulating presumptive Purkinje cell axons. The i.p.s.cs were reversibly abolished by bicuculline (10 μM ; data not shown), indicating that responses were mediated by GABA (γ -aminobutyric acid). Bath application of synthetic ω -Aga-IVA (200 nM) reduced i.p.s.c. amplitude to ~2% of control within a few minutes (Figs 1a, d and 2i; Table 1); this effect was irreversible. In contrast to evoked i.p.s.cs, the amplitude of spontaneous miniature i.p.s.cs remained the same ($96 \pm 4.2\%$, mean $\pm$ s.e.m.; 7 cells) after application of the toxin, indicating that its site of action is presynaptic. The inhibitory effect of ω -Aga-IVA was dose-dependent (Fig. 1d), with a 50%-inhibition dose (IC_{50}) of ~10 nM. ω -CgTx, an N-type Ca^{2+} blocker, also suppressed the i.p.s.cs (Figs 1b and 2i) in a dose-dependent manner, with a maximum inhibition of ~50% and an IC_{50} of ~0.1 μM (Fig. 1e; Table 1). This effect of ω -CgTx was also irreversible. By contrast, the L-

TABLE 1 Synaptic current fractions remaining after application of Ca^{2+} -channel-blocking toxins

Toxins applied	ω -Aga-IVA (a)	ω -CgTx (b)	ω -Aga-IVA + ω -CgTx (c)	a+b+c
Cerebellar i.p.s.cs	0.02 ± 0.01 (0.73)	0.50 ± 0.07 (0.21)	0.005 ± 0.001 (0.17)	1.11
Spinal i.p.s.cs	0.09 ± 0.06 (0.55)	0.51 ± 0.09 (0.20)	0.01 ± 0.003 (0.22)	0.97
Hippocampal e.p.s.cs	0.16 ± 0.02 (0.46)	0.69 ± 0.07 (0.12)	0.07 ± 0.02 (0.41)	0.99

Each value represents mean ($\pm$ s.e.m.) of six cells. Numbers in parentheses (a–c) are the estimated fractions of Ca^{2+} -channel subtypes mediating synaptic transmission, assuming a third power relation between presynaptic Ca^{2+} concentration and postsynaptic response amplitude.

type Ca^{2+} channel blocker nifedipine (10 μM) had virtually no effect (Figs 1c and 2i).

There was an apparent overlap in the effect of ω -Aga-IVA and ω -CgTx on the i.p.s.cs. Inhibitory postsynaptic currents were inhibited by ω -Aga-IVA by 98%, and also by ω -CgTx by 50% (Fig. 1; Table 1). This overlap can be explained by the nonlinear relationship between presynaptic calcium concentration and transmitter release described at various synapses^{1,14–16}. With a nonlinear relationship, only a small decrease in presynaptic Ca^{2+} entry can cause a large reduction in the synaptic responses. Assuming a power function for the relationship, the relative amplitude of postsynaptic currents remaining after application of ω -Aga-IVA (A), ω -CgTx (B) or both toxins (C) can be described as $A = (1-a)^m$, $B = (1-b)^m$ and $C = c^m$, where a , b and c represent a fraction of presynaptic Ca^{2+} channel subtypes sensitive to ω -Aga-IVA (a) and ω -CgTx (b) and those insensitive to the toxins (c) respectively, and m is the power coefficient. If each toxin blocks only one type of presynaptic Ca^{2+} channel, $a+b+c$ should be unity. In our estimation from the data obtained from different synapses (see later), the range of the values was 0.97–1.11 (Table 1) or 0.92–1.05, assuming $m = 3$ or $m = 4$ (refs 14–16). Thus, the apparent overlap in the blockade of synaptic currents by the two toxins is likely to be due to the power relationship between presynaptic Ca^{2+} and transmitter release.

The marked blockade by ω -Aga-IVA of i.p.s.cs recorded from cells in the cerebellar nuclei is consistent with a predominant occurrence of P-type Ca^{2+} channels in Purkinje cells^{5,10}. We investigated whether synaptic responses in other neurons could be blocked by ω -Aga-IVA. Inhibitory postsynaptic currents were evoked in dorsal horn neurons of rat spinal cord by stimulating neighbouring interneurons¹⁶. i.p.s.cs were identified as gly-

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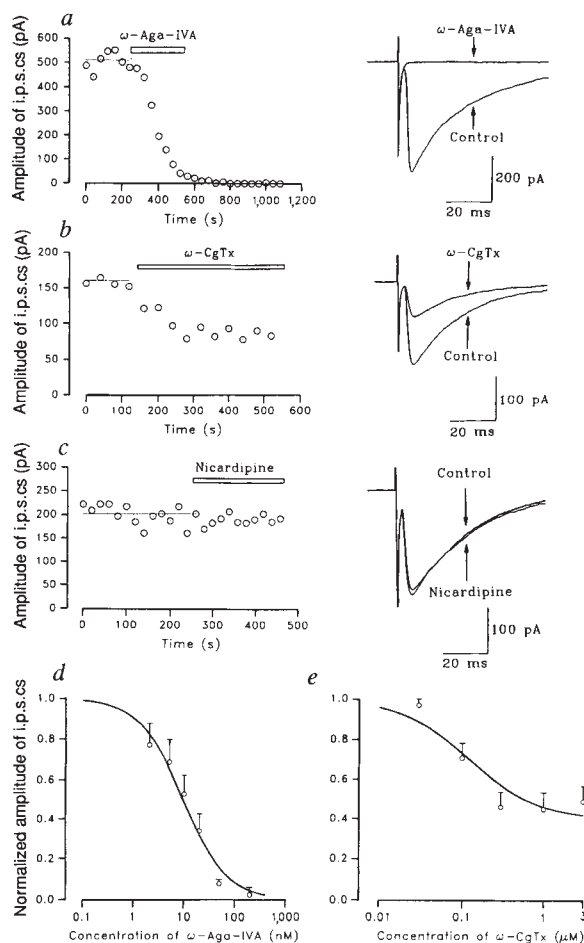
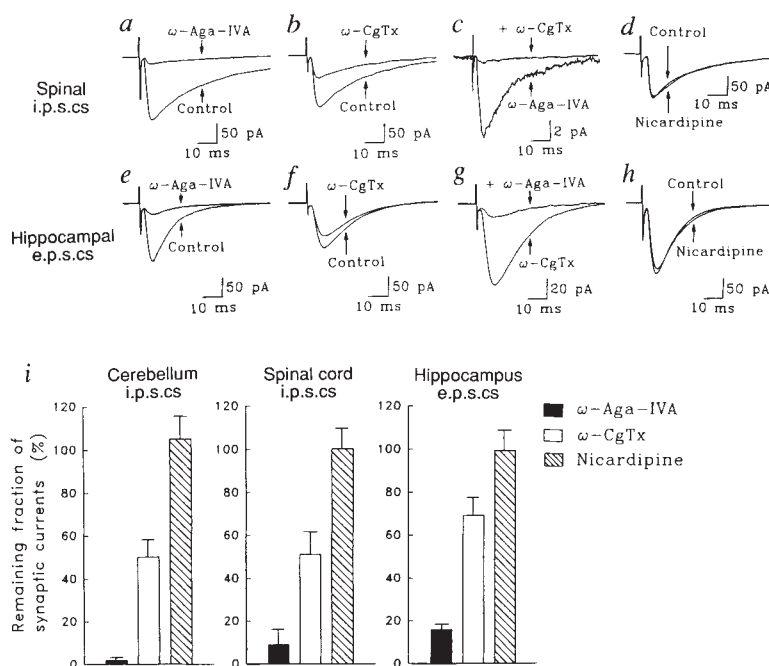


FIG. 1 Effects of Ca^{2+} channel blockers on GABAergic i.p.s.cs in cerebellar nuclear cells. *a-c*, Effects of ω -Aga-IVA (200 nM; *a*), ω -CgTx (3 μM ; *b*) and nicardipine (10 μM ; *c*) on the amplitude of i.p.s.cs (ordinates). Blockers were applied during the periods indicated by horizontal bars. From 10 to 20 i.p.s.cs were averaged for each data point. Lines indicate the mean amplitude of i.p.s.cs before drug application. Insets, averaged i.p.s.cs at 420 s (*a*), 320 s (*b*) and 140 s (*c*) after drug application are superimposed on those before drug application. *d* and *e*, Dose-dependency of the i.p.s.cs-blocking effects of ω -Aga-IVA (*d*) and ω -CgTx GVIA (*e*). Ordinates show the amplitude of remaining i.p.s.cs after toxin application normalized to control. Means and standard errors of mean (s.e.m., 6 cells) are indicated. Data points were fitted using the χ^2 method and equations $y=1/(1+x/\text{IC}_{50})$ for *d*, and $y=0.40+0.60/(1+x/\text{IC}_{50})$ for *e* (x and y, abscissa and ordinate). The IC_{50} values were 9.4 nM and 0.13 μM , respectively, for *d* and *e*.

METHODS. Sagittal slices 130–150 μm thick were prepared from cerebellum of 6–8-day-old rats⁷. The slices were superfused with Krebs solution¹⁶ bubbled with O_2/CO_2 (95/5%). Whole-cell recordings were made from visually identified cerebellar nuclear cells (cell capacitance, 37.2 ± 1.5 pF (mean $\pm$ s.e.m.); 82 cells) with a thin-wall borosilicate glass patch pipette (resistance, 4.2–6.7 M Ω) filled with an internal solution (in mM): 140 CsCl, 9 NaCl, 1 MgCl₂, 1 EGTA, 10 HEPES (pH 7.3, adjusted with KOH). Series resistance (10–25 M Ω) was continuously monitored and occasionally compensated for a slight change during recordings. Stimulation was made with a glass pipette¹⁶ positioned in the vicinity of Purkinje cell axons. GABAergic i.p.s.cs were evoked in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and strychnine (0.5 μM). ω -Aga-IVA and ω -CgTx (Peptide Institute) were dissolved in an oxygenated Krebs solution containing cytochrome c (1 mg ml⁻¹) just before bath application. Cytochrome c did not itself affect the synaptic response (not shown). Perfusion rate was maintained at 2.2–2.5 ml min⁻¹ and the bath volume was ~ 1.7 ml. The switching solution was made manually, with a dead space corresponding to 80 s. All experiments were done under voltage-clamp at a holding potential of -70 mV at room temperature (20–24 $^{\circ}\text{C}$). Synthetic ω -Aga-IVA was a gift from T. Kimura, Peptide Institute, Osaka. As previously reported^{5,8}, ω -Aga-IVA blocked most of the Ca^{2+} -channel current recorded from Purkinje cells in slices (not shown). Nicardipine (Sigma) was diluted from a 10 mM stock solution in dimethyl sulphoxide. The control solution for nicardipine application contained dimethyl sulphoxide at a final concentration of 0.1%. Nicardipine was protected from light. Significance in difference was evaluated by Student's *t*-test, with 5% confidence limit.

FIG. 2 Effects of Ca^{2+} channel blockers on i.p.s.cs in spinal neurons and e.p.s.cs in hippocampal neurons. *a-d*, Glycinergic i.p.s.cs recorded from spinal neurons. Averaged i.p.s.cs before and after applications of ω -Aga-IVA (200 nM; *a*), ω -CgTx (3 μM ; *b*) and nicardipine (10 μM ; *d*) were superimposed. In *c*, an averaged i.p.s.c. after ω -Aga-IVA application was superimposed with that after further application of ω -CgTx (stimulus artefacts were reduced by subtracting records with failure from those with responses). *e-h*, Non-NMDA glutamatergic e.p.s.cs recorded from hippocampal CA1 pyramidal cells. The e.p.s.cs were reversibly blocked by CNQX (10 μM ; results not shown). Averaged e.p.s.cs before and after applications of ω -Aga-IVA (200 nM; *e*), ω -CgTx (3 μM ; *f*) and nicardipine (10 μM ; *h*) were superimposed. In *g*, an averaged i.p.s.c. after ω -CgTx application was superimposed on that after further application of ω -Aga-IVA. *i*, Remaining fractions of synaptic currents after application of Ca^{2+} -channel blockers. Columns and bars represent mean amplitude and s.e.m. of synaptic currents normalized with control before drug application. Values for nicardipine are $105 \pm 9.1\%$ (6 cells), $100 \pm 4.3\%$ (7 cells) and $99 \pm 8.0\%$ (6 cells) in cerebellar, spinal i.p.s.cs and hippocampal e.p.s.cs, respectively (see Table 1 for other values).

METHODS. Slices (120 μm thick) were made from lumbar spinal cord of 4–8-day-old rats. Glycinergic i.p.s.cs were evoked in dorsal neurons (28 ± 2.0 pF; 26 cells) by stimulating neighbouring neurons at 0.5–2 Hz in the presence of CNQX (5 μM) and bicuculline (10 μM)¹⁶. Slices (150 μm thick) were made from hippocampal CA1 pyramidal neurons (34 ± 1.7 pF, 25 cells) of 14–16-day-old rats. e.p.s.cs were evoked by stimulating Schaffer collaterals at stratum radiatum in the presence



of (+)-2-amino-5-phosphonovalerate (50 μM), bicuculline (10 μM) and strychnine (0.5 μM). 30–60 records were averaged for traces in *a-h*.

cinergic from their sensitivity to strychnine. These i.p.s.cs were almost entirely blocked by ω -Aga-IVA (200 nM; Fig. 2a, i), and less markedly by ω -CgTx (3 μ M; Fig. 2b, i). Nicardipine (10 μ M) had no effect (Fig. 2d, i). Small i.p.s.cs remaining after ω -Aga-IVA application were blocked by ω -CgTx (Fig. 2c; note calibration). Only about 1% of i.p.s.cs remained after application of both toxins (Table 1). A similar result was obtained for glutamatergic excitatory postsynaptic currents (e.p.s.cs) evoked in hippocampal CA1 pyramidal cells by stimulating Schaffer collaterals (Fig. 2e-h). A marked suppression of the e.p.s.cs by ω -Aga-IVA (Fig. 2e, i) was rather unexpected, because the density of ω -Aga-IVA-sensitive Ca^{2+} channels in CA3 cell somata is relatively low⁵. In agreement with previous reports^{7,8}, the hippocampal e.p.s.cs were partially suppressed by ω -CgTx (Fig. 2f, i), but not affected by nicardipine (Fig. 2h, i). The e.p.s.cs remaining after ω -CgTx application were further blocked by ω -Aga-IVA (Fig. 2g), leaving about 7% of the control response (Table 1).

Our results show that both ω -Aga-IVA-sensitive and ω -CgTx-sensitive Ca^{2+} channels mediate neurotransmitter release in the central nervous system (CNS). These two classes of Ca^{2+} channel probably correspond to the Ca^{2+} channels previously classified as P-type and N-type^{5,17,18}. The use of ω -Aga-IVA for pharmacological identification, however, leaves open the possibility that synaptic transmission is mediated by other Ca^{2+} -channel types that are sensitive to this toxin, such as the recently cloned B1 (refs 11, 19) or rB-E-II channels¹². The IC_{50} value of ω -Aga-IVA for cerebellar i.p.s.cs (10 nM) was higher than that reported for P-type Ca^{2+} channels (2 nM)⁵. The discrepancy between the IC_{50} values may be even greater, given the power relation between Ca^{2+} entry and transmitter release. This raises the possibility that the ω -Aga-IVA-sensitive Ca^{2+} channels in the presynaptic terminals may be heterogeneous.

Our data are consistent with several types of Ca^{2+} channel being present in the active zone. Co-localization of different types of Ca^{2+} channels in the active zone may be important for synaptic function. The synaptic facilitation and depression produced by successive presynaptic volleys have different time

courses at different synapses²⁰, depending on the changes in presynaptic Ca^{2+} concentration²¹. This variability might be due, in part, to variable proportions of presynaptic Ca^{2+} channels having different inactivation kinetics, such as N- and P-type Ca^{2+} channels⁵. Co-existence of two types of Ca^{2+} channel with distinct inactivation kinetics has also been reported for peptide-releasing nerve terminals in rat neurohypophysis²². Ca^{2+} -channel regulation is thought to be important in the control of synaptic efficacy by alteration of transmitter release probability²³⁻²⁵. The presence of different types of presynaptic Ca^{2+} channels with different regulatory sites would permit great flexibility in transmitter release at a single synapse. □

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Molecular determinants of Ca^{2+} selectivity and ion permeation in L-type Ca^{2+} channels

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VOLTAGE-GATED Ca^{2+} channels link changes in membrane potential to the delivery of Ca^{2+} , a key second messenger for many cellular responses¹. Ca^{2+} channels show selectivity for Ca^{2+} over more plentiful ions such as Na^{+} or K^{+} by virtue of their high-affinity binding of Ca^{2+} within the pore²⁻⁶. It has been suggested that this binding involves four conserved glutamate residues⁷⁻¹⁰ in equivalent positions in the putative pore-lining regions of repeats I-IV in the Ca^{2+} channel α_1 subunit. We have carried out a systematic series of single amino-acid substitutions in each of these positions and find that all four glutamates participate in high-affinity binding of Ca^{2+} or Cd^{2+} . Each glutamate carboxylate makes a distinct contribution to ion binding, with the carboxylate in repeat III having the strongest effect. Some single glutamate-to-lysine muta-

tions completely abolish micromolar Ca^{2+} block, indicating that the pore does not possess any high-affinity binding site that acts independently of the four glutamate residues. The prevailing model of Ca^{2+} permeation^{2,3} must thus be modified to allow binding of two Ca^{2+} ions in close proximity^{11,12}, within the sphere of influence of the four glutamates. The functional inequality of the glutamates may be advantageous in allowing simultaneous interactions with multiple Ca^{2+} ions moving single-file within the pore. Competition among Ca^{2+} ions for individual glutamates^{11,12}, together with repulsive ion-ion electrostatic interaction^{2,3}, may help achieve rapid flux rates through the channel²⁻⁵.

Figure 1a aligns amino-acid sequences within the putative pore-lining regions (SS1-SS2) of each of the four internal repeats of the cardiac L-type Ca^{2+} channel α_1 subunit¹³. The four glutamate residues indicated (residues 393, 736, 1,145 and 1,446), here designated as E_I, E_{II}, E_{III} and E_{IV}, seem to be conserved among all voltage-gated Ca^{2+} channels^{7,9}. The importance of these residues has been suggested by work on Na^{+} channels, in which the homologous residues in repeats I-IV are Asp, Glu, Lys and Ala respectively. Replacement of Lys and Ala with glutamates endowed a Na^{+} channel with ion-selection properties qualitatively similar to those of Ca^{2+} channels⁹. To characterize the functional role of all four glutamates in ion selectivity and permeation in a voltage-gated Ca^{2+} channel, we constructed point mutants in each of the four repeats, replacing glutamate with either glutamine or lysine. Whole-cell currents were recorded from *Xenopus* oocytes injected with complementary RNA encoding the wild-type (WT) or mutant channel proteins.

The interactions of the L-type channels with Ca^{2+} were first

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assessed by examining Ca^{2+} block of inward Ba^{2+} currents (I_{Ba}). Figure 1b shows representative records of Ba^{2+} currents from wild type and the mutants E_1Q and $\text{E}_{\text{III}}\text{Q}$. In wild type, 3 mM Ca^{2+} reduced the current by $56 \pm 1\%$ (mean $\pm$ s.e.m., $n=5$). Whereas the single $\text{E} \rightarrow \text{Q}$ mutation in repeat I exerted little effect, mutations in corresponding residues in repeats II, III and IV attenuated Ca^{2+} block. The severity of the mutations followed the order $\text{E}_{\text{III}}\text{Q} > \text{E}_{\text{IV}}\text{Q} > \text{E}_{\text{II}}\text{Q} > \text{E}_1\text{Q} > \text{WT}$. Thus, the contributions of the four glutamates to Ca^{2+} interactions are not equivalent.

In the absence of divalent cations, Ca^{2+} channels carry large monovalent ion currents^{2-6,11,14,15}. Ca^{2+} block of such current provides a more direct index of high-affinity Ca^{2+} binding to the pore. Figure 1c illustrates inward Li^+ current (I_{Li}) in the absence or presence of 1 μM Ca^{2+} , for wild type, E_1Q and $\text{E}_{\text{III}}\text{Q}$. The Li^+ currents were carried by exogenous L-type Ca^{2+} channels, not endogenous channels, as they could be inhibited completely by the L-type antagonist nifedipine (50 μM ; data not shown). Ca^{2+} blocked I_{Li} in the wild-type channel with an IC_{50} (concentration giving 50% inhibition) of 0.97 μM (Fig. 1d), about 3,000-fold less than required for 50% block of I_{Ba} . All four single $\text{E} \rightarrow \text{Q}$ mutations reduced Ca^{2+} block of I_{Li} (Fig. 1d). As with block of I_{Ba} , these mutations affected block of I_{Li} unequally: $\text{E}_{\text{III}}\text{Q}$ increased IC_{50} by almost 20-fold, whereas E_1Q increased IC_{50} by only 2-fold. The order of IC_{50} s was $\text{E}_{\text{III}}\text{Q} > \text{E}_{\text{IV}}\text{Q} > \text{E}_1\text{Q} > \text{WT}$. This pattern of Ca^{2+} block is similar but not identical to that found with Ba^{2+} as the permeating ion.

In a double mutant, $\text{E}_1\text{Q} \cdot \text{E}_{\text{IV}}\text{Q}$, Ca^{2+} block was greatly reduced, with an IC_{50} of 104 μM (Fig. 1d). This is >100-fold greater than in wild type, much larger than the 14-fold change

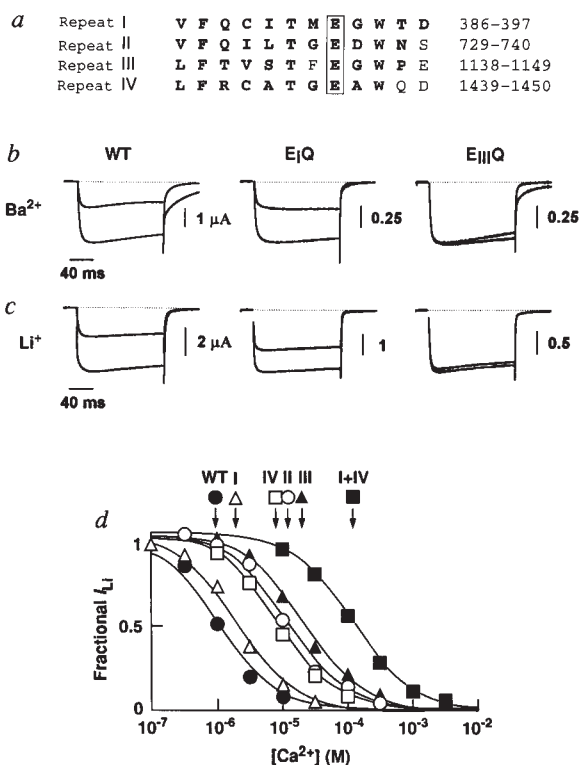
(1.85×7.7) predicted for additive changes in binding energy. Thus, the individual mutations do not contribute independently to Ca^{2+} binding (compare ref. 16).

Cadmium ions were used as an additional probe for the molecular determinants of divalent cation binding. Cd^{2+} competes with Ca^{2+} for high-affinity binding to Ca^{2+} channels^{5,11,17}, but differs in its coordination chemistry: Cd^{2+} prefers a tetrahedral ligand geometry whereas Ca^{2+} exhibits less strict coordination requirements¹⁸. Dose-response relationships (Fig. 2) indicate substantial differences in sensitivity to Cd^{2+} block for each of the four $\text{E} \rightarrow \text{Q}$ mutants relative to wild type. For I_{Ba} , IC_{50} values for the mutants ranged from 2- to 37-fold higher than wild type (Fig. 2a); for I_{Li} , the mutations were even more powerful, increasing the IC_{50} for Cd^{2+} block by 13- to 470-fold (Fig. 2b). Regardless of the charge carrier, the rank order of potency was $\text{E}_{\text{III}}\text{Q} > \text{E}_1\text{Q} > \text{E}_{\text{II}}\text{Q} > \text{E}_{\text{IV}}\text{Q} > \text{WT}$. This order of effectiveness for Cd^{2+} block differs substantially from the ranking for Ca^{2+} block, where E_1Q was by far the least effective mutation (Fig. 1). In the double mutant, $\text{E}_1\text{Q} \cdot \text{E}_{\text{IV}}\text{Q}$, Cd^{2+} block of I_{Ba} was greatly weakened ($\text{IC}_{50} = 42 \mu\text{M}$).

Glutamine is a polar residue capable of serving as a ligand for Ca^{2+} binding in other proteins¹⁸. Thus, one might expect that effects of single $\text{E} \rightarrow \text{Q}$ mutations would be surpassed by replacement of glutamate with a positively charged residue such as lysine. Figure 3a displays the effects of single $\text{E} \rightarrow \text{K}$ mutations on Li^+ or Ba^{2+} currents. All four lysine mutants carried significant I_{Li} . However, lysine mutations in each of the first three repeats gave negligible I_{Ba} ; $\text{E}_{\text{IV}}\text{K}$ supported a significant I_{Ba} , albeit smaller than the I_{Li} recorded from the same cell. The results illustrated for $\text{E}_{\text{II}}\text{K}$ are representative of E_1K and $\text{E}_{\text{III}}\text{K}$

FIG. 1 Mutations in the SS2 regions attenuate Ca^{2+} block of Ba^{2+} or Li^+ currents. a, Amino-acid sequences in the SS1-SS2 regions of the four repeats in the cardiac L-type Ca^{2+} channel¹³. Residues conserved among all known voltage-gated Ca^{2+} channels are indicated in boldface, with residue numbers given on the right. Single amino-acid substitutions introduced at the boxed Glu residues ($\text{E} \rightarrow \text{Q}$ or $\text{E} \rightarrow \text{K}$) are denoted as E_1Q or E_1K , $\text{E}_{\text{II}}\text{Q}$, or $\text{E}_{\text{II}}\text{K}$, and so on. b, Whole-cell Ba^{2+} currents evoked by a depolarizing test pulse from a holding potential of -60 mV to +10 mV in the absence or presence of 3 mM external Ca^{2+} for WT, E_1Q and $\text{E}_{\text{III}}\text{Q}$. Tail currents resulted from Ca^{2+} influx causing activation of an endogenous Ca^{2+} -dependent chloride channel in the oocyte. The average block of I_{Ba} by 3 mM Ca^{2+} was $5 \pm 1.1\%$, $11.6 \pm 0.5\%$, $34.5 \pm 0.3\%$, $55 \pm 0.7\%$ and $56 \pm 1.0\%$ (mean $\pm$ s.e.m.; $n=4$ or 5) in $\text{E}_{\text{III}}\text{Q}$, $\text{E}_{\text{IV}}\text{Q}$, $\text{E}_{\text{II}}\text{Q}$, E_1Q and WT, respectively. c, Whole-cell Li^+ currents evoked by a depolarizing pulse from -60 mV to -20 mV in Ca^{2+} -free control solution and in the presence of 1 μM external Ca^{2+} . d, Dose-response relationships for Ca^{2+} block of inward Li^+ currents. Averaged fractional peak currents ($n=4-8$) were plotted as a function of external Ca^{2+} concentration; s.e.m. is smaller than the symbols in all cases. IC_{50} s indicated by the downward arrows at the top are 0.97, 1.85, 7.7, 10.7, 18.6 and 104 μM for WT, E_1Q , $\text{E}_{\text{IV}}\text{Q}$, $\text{E}_{\text{II}}\text{Q}$, $\text{E}_{\text{III}}\text{Q}$ and $\text{E}_1\text{Q} \cdot \text{E}_{\text{IV}}\text{Q}$. Other mutant channels with amino-acid substitutions at nearby positions (D377N, D397N, D404N, D737N, E1464Q and E1466Q) were also tested and showed no increase in the IC_{50} for Ca^{2+} block of inward Li^+ currents relative to wild type.

METHODS. Individual point mutations were introduced into the rabbit cardiac L-type Ca^{2+} channel¹³ using standard techniques. Wild-type and mutant complementary DNA constructs were linearized with *Xba*I and transcribed *in vitro* with either SP6 or T7 polymerase. Oocytes prepared from *Xenopus laevis* were injected with ~50 nl of a cRNA mixture which contained equimolar ratios of the α_1 ($0.1 \mu\text{g} \mu\text{l}^{-1}$)¹³, α_2 ($0.1 \mu\text{g} \mu\text{l}^{-1}$)²⁷ and β_{2b} ($0.03 \mu\text{g} \mu\text{l}^{-1}$)²⁸ subunits. Cells were incubated at 18 °C for 4-8 days before recording. Whole-cell currents recorded at room temperature (~20-23 °C) using a standard two-electrode voltage-clamp method²⁹ were filtered at 500 Hz and digitized at 2 kHz. Currents were elicited every 15 s by 150-ms depolarizing test pulses to either -20 or +10 mV from a holding potential of -60 mV, and were leak-subtracted by a P/4 model. Dose-response curves were generated according to the one-to-one binding equation: $I/I_{\text{con}} = 1/(1 + [X]/\text{IC}_{50})$, where $[X]$ is the concentration of the blocking ion. The Ba^{2+} external solutions contained 40 mM $\text{Ba}(\text{OH})_2$, 52 mM TEA-OH, 5 mM HEPES, pH adjusted to 7.4 with $\text{CH}_3\text{SO}_3\text{H}$. For experiments in b, 3 mM Ca^{2+} was added to the testing solutions. The control Li^+ bathing solution contained 100 mM



LiCl , 10 mM HEPES, 10 mM H-EDTA, 14 mM TEA-Cl, 15 μM CaCl_2 , pH adjusted with TEA-OH to 7.4, and had an osmolality of ~256 mmol kg^{-1} . Free Ca^{2+} concentration in the solution was ~3 nM. Li^+ solutions containing the appropriate concentrations of free Ca^{2+} (up to 30 μM) were made by adding appropriate amounts of CaCl_2 to the control solution; constant osmolality was maintained by decreasing the amount of TEA-Cl. pH was readjusted to 7.4 with TEA-OH. In solutions containing 100 μM or more free Ca^{2+} , H-EDTA was omitted and appropriate amounts of CaCl_2 and TEA-Cl were added.

as well. The Ba^{2+} current was carried through the $\text{E}_{\text{IV}}\text{K}$ mutant channels as it could be greatly augmented by the L-type Ca^{2+} channel agonist FPL 64176 (1 μM ; ref. 19).

Current-voltage relationships in both Ba^{2+} and Li^{+} solutions (Fig. 3b) were strongly affected by the lysine mutations. Reversal potentials (E_{rev}) in Ba^{2+} solution shifted from $+67 \pm 0.3$ mV in wild type ($n=7$) to $+35 \pm 1.3$ mV in $\text{E}_{\text{IV}}\text{K}$ ($n=3$), and to less than $+5$ mV in the other mutants ($n=3-9$), indicating that the permeability to Ba^{2+} was reduced only moderately in $\text{E}_{\text{IV}}\text{K}$, but drastically in the other mutants. Thus, the Ca^{2+} channel seems to have become more monovalent-cation-selective than divalent-cation-selective as a result of the $\text{E} \rightarrow \text{K}$ mutations in repeats I, II or III. The lysine mutations produced additional alterations in selectivity among monovalent cations. Whereas E_{rev} with external Li^{+} was $+2.5 \pm 1.1$ mV in wild type ($n=7$), it was $+27 \pm 0.9$ mV in $\text{E}_{\text{II}}\text{K}$ ($n=4$) and $+8.7 \pm 1.3$ mV in $\text{E}_{\text{III}}\text{K}$ ($n=8$). In contrast, the mutants $\text{E}_{\text{I}}\text{K}$ and $\text{E}_{\text{IV}}\text{K}$ showed little difference from wild type ($n=4$). Thus, the permeability ratio $P_{\text{Li}}/P_{\text{K}}$ increased about 3-fold for $\text{E}_{\text{II}}\text{K}$ relative to wild type, slightly for $\text{E}_{\text{III}}\text{K}$, but was not detectably changed for the other mutants.

The potency of Ca^{2+} block of Li^{+} current was greatly reduced (100- to 1,000-fold) by each of the four $\text{E} \rightarrow \text{K}$ mutations (Fig. 3c). The impact of the amino-acid substitutions varied from

repeat to repeat, as in the $\text{E} \rightarrow \text{Q}$ mutations, although the order of effectiveness was again different. In this case, the $\text{E}_{\text{II}}\text{K}$ mutation ($\text{IC}_{50} = 0.81$ mM) almost matched $\text{E}_{\text{III}}\text{K}$ ($\text{IC}_{50} = 1.1$ mM); high-affinity Ca^{2+} block was eliminated in both cases.

These experiments demonstrate the fundamental importance of the glutamate residues in SS2 for ion selectivity, permeation and high-affinity Ca^{2+} binding in L-type Ca^{2+} channels. As the SS2 glutamates seem perfectly conserved among all known α_1 subunits, our conclusions about Ca^{2+} interactions with L-type channels may hold for voltage-gated Ca^{2+} channels in general. Our results and those of others^{10,20} support the identification of SS1-SS2 as a constituent of the Ca^{2+} channel pore, as previously found for Na^{+} and K^{+} channels^{7-9,21-23}.

Mutations within the set of glutamates were effective in reducing the pore's affinity for Ca^{2+} by as much as 10^3 -fold, raising the IC_{50} for Ca^{2+} block of I_{Li} from ~ 1 μM to ~ 1 mM. Evidently no other high-affinity Ca^{2+} binding site exists independently of the four glutamates. There was no hint of an external regulatory site mediating micromolar Ca^{2+} block¹⁴, nor of a second, discrete, high-affinity site at the other end of the pore^{2,3}. Instead, our results suggest that all high-affinity divalent cation interactions take place within the sphere of influence of the four glutamate residues. Thus, we must modify the prevailing hypothesis for Ca^{2+} channel permeation^{2,3}, placing the high-affinity Ca^{2+} binding sites closer together, to the extent that they may even share some of the same ligands; this is reminiscent of recent proposals^{11,12}. Together these studies support the following

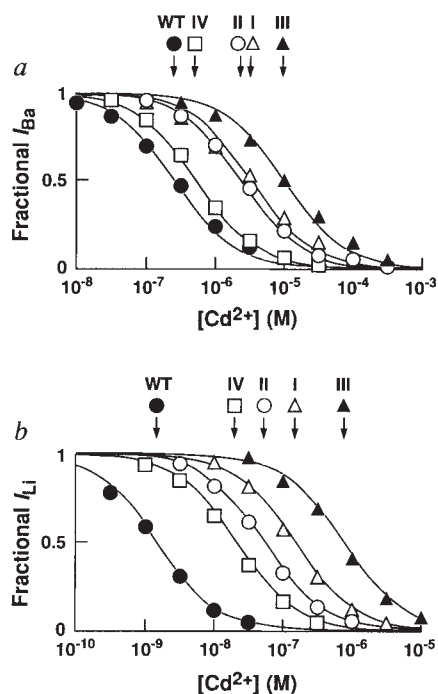


FIG. 2 Single $\text{E} \rightarrow \text{Q}$ mutations reduce Cd^{2+} block of both Ba^{2+} and Li^{+} currents. Fractional peak Ba^{2+} (a) or Li^{+} (b) currents plotted against external Cd^{2+} concentration as indicated. Symbols (same as in Fig. 1d) represent the average of 4-6 experiments; s.e.m. is smaller than the symbols in all cases. Smooth curves are the least-squares fits to the one-to-one binding equation in Fig. 1. IC_{50} s indicated by downward arrows at the top are in the order of WT, $\text{E}_{\text{IV}}\text{K}$, $\text{E}_{\text{II}}\text{K}$, $\text{E}_{\text{I}}\text{K}$ and $\text{E}_{\text{III}}\text{K}$, with values of 0.26, 0.53, 2.4, 3.3 and 9.7 μM for block of Ba^{2+} current, and 1.4, 18, 48, 132 and 658 nM for block of Li^{+} current.

METHODS. The external Li^{+} solution used in b contained 100 mM LiCl, 10 mM HEPES, 25 mM TEA-Cl, pH adjusted to 7.4 with TEA-OH. To remove contaminating Ca^{2+} and other divalent cations, the solution was passed through a 'Ca²⁺ sponge' prepared from a heptadentate diethylene-triaminepentaacetic acid anhydride-based resin that strongly binds Ca^{2+} (ref. 30). The free Ca^{2+} concentration in the 'sponged' solution was similar to that in the control Li^{+} solution used in Fig. 1 as the amplitude of I_{Li} recorded from individual cells was similar in both solutions. Cd^{2+} was added to the 'sponged' Li^{+} solution at desired concentrations.

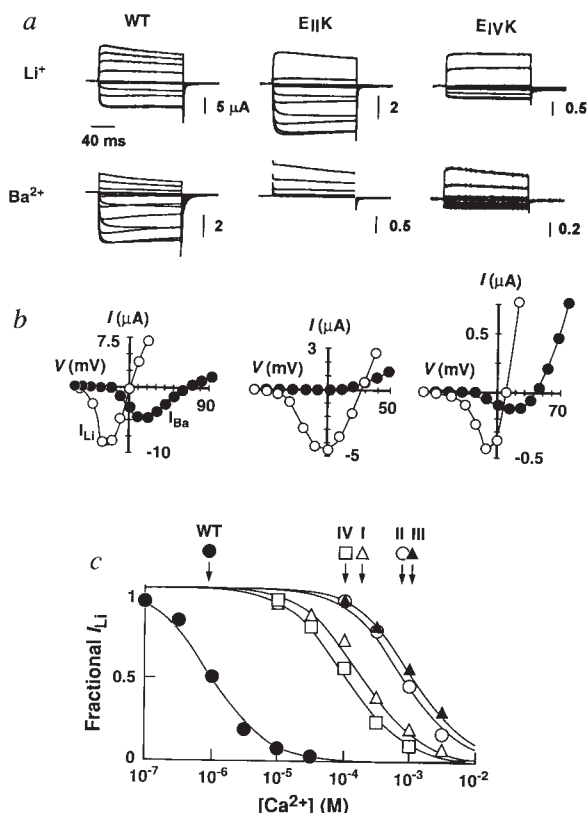


FIG. 3 Single $\text{E} \rightarrow \text{K}$ mutations affect ion permeation and Ca^{2+} binding. a, Families of whole-cell currents recorded from oocytes expressing the wild-type and $\text{E}_{\text{II}}\text{K}$, $\text{E}_{\text{IV}}\text{K}$ mutant channels with external solutions containing either Li^{+} or Ba^{2+} as the only inward current charge carrier. b, Current-voltage (I - V) relationships in Ba^{2+} (●) or Li^{+} (○) solutions. c, Dose-response relationships for Ca^{2+} block of inward Li^{+} currents. Averaged fractional peak currents ($n=4-6$) were plotted as a function of external Ca^{2+} concentration. s.e.m. is smaller than the symbols in all cases. IC_{50} s indicated by downward arrows at the top are 0.97, 109, 195, 819 and 1,114 μM for WT, $\text{E}_{\text{IV}}\text{K}$, $\text{E}_{\text{I}}\text{K}$, $\text{E}_{\text{II}}\text{K}$ and $\text{E}_{\text{III}}\text{K}$.

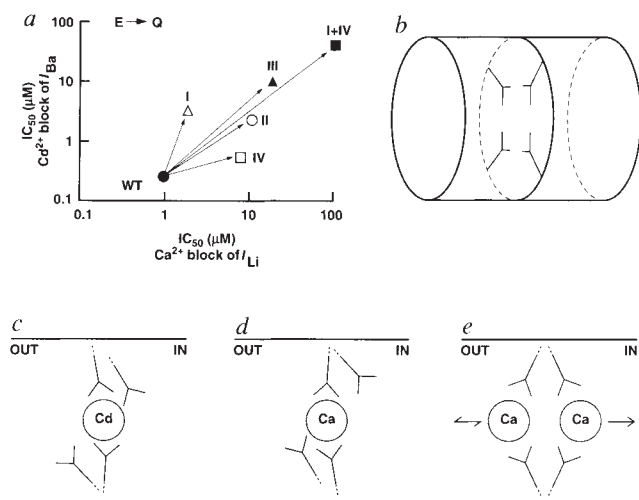


FIG. 4 Functional inequality of the four glutamate residues in the L-type Ca^{2+} channel and models of their spatial organization. **a**, Summary diagram of two key functional properties of four single E→Q mutants and the double mutant, E₁Q·E₄Q. The IC_{50} of Cd^{2+} block of I_{Ba} (ordinate) is plotted against the IC_{50} of Ca^{2+} block of I_{Li} (abscissa) for WT and each mutant. The arrows indicate the functional effect of the structural change from WT to mutant. Same symbols as in Fig. 1d. **b**, Schematic drawing of a strictly symmetrical arrangement of the four glutamate carboxylates (represented by the forks), all lying within a common plane perpendicular to the permeation pathway. **c** and **d**, Asymmetrical model with carboxylates contributing unequally to high-affinity binding of divalent cations such as Cd^{2+} (**c**) or Ca^{2+} (**d**). Inequality of the glutamates may arise from asymmetry in their geometrical arrangement or in their local environment (see text). Binding configurations for Cd^{2+} (**c**) and Ca^{2+} (**d**) are hypothesized to involve individual carboxylates to different extents. **e**, Schematic representation of the proposal that the four glutamates can also contribute to the accommodation of two Ca^{2+} ions simultaneously. The doubly occupied state is attained when a Ca^{2+} ion enters from the outside and competes with a previously bound Ca^{2+} ion for carboxylate groups. Sharing of ligands and possible electrostatic repulsion between the two Ca^{2+} ions strongly diminish their binding affinity and thereby promote net influx of Ca^{2+} into the cell (inward-pointing arrow).

hypothesis for Ca^{2+} channel selectivity and ion permeation. All four glutamate carboxylates can contribute collectively to high-affinity binding of a single divalent cation (Fig. 4c, d), thus obstructing flow of monovalent cations in the single-file pore and providing strong selectivity for Ca^{2+} over Na^{+} or K^{+} under physiological conditions. The set of glutamates can also interact simultaneously with two Ca^{2+} ions (Fig. 4e), but in this case the affinity for both ions is greatly weakened (apparent $K_d \sim 14 \text{ mM}$; ref. 4). The weaker interactions with multiple divalent cations, key to rapid ion dissociation and flux, might arise from electrostatic repulsion between ions^{2,3} and/or ion competition for common ligands^{11,12}. Both kinds of negative interactions would be enhanced by the close proximity of the Ca^{2+} ions as they interact with the glutamates.

Our experiments have interesting structural implications. It is clear that high-affinity Ca^{2+} -binding arises from carboxylate side-chains provided by multiple internal repeats of the Ca^{2+} channel, thus resembling the Ca^{2+} -ATPase²⁴ rather than EF-hand proteins, whose Ca^{2+} binding sites are localized to individual domains²⁵. It is striking that replacements of individual carboxylates produce such different degrees of functional alteration, given the generally fourfold architecture of Ca^{2+} channels, and the high homology among the four SS1–SS2 regions. Distinctions between the four glutamates are extended by comparison of their contributions to Ca^{2+} and Cd^{2+} block (Fig. 4a). The distinct contributions of each of the glutamates to high-affinity divalent cation binding would not be expected if the pore contained a symmetrical ring of four strictly equivalent glutamates (Fig. 4b). Thus, Ca^{2+} channels may differ from cer-

tain K^{+} channels^{7,8} and neuronal nicotinic acetylcholine receptor channels²⁶, which seem capable of operating with symmetrical rings of amino-acid residues. The non-equivalence of the Ca^{2+} channel glutamates might arise from asymmetry in the positions of their α -carbon atoms, or in the orientation or negativity of their carboxylate side chains, possibly under the influence of nonequivalent residues nearby (Fig. 1a; ref. 10). Figure 4c and d illustrates one hypothetical arrangement, which positions key carboxylates at non-identical axial locations along the conduction path¹¹. This provides favourable geometry for carboxylate interactions with multiple ions moving single-file through the pore, and also allows a tentative interpretation of the unequal contributions of the glutamates. E₁Q was the only glutamate within this set whose replacement by lysine allowed substantial Ba^{2+} current, suggesting that its side chain is the closest to the external pore entrance. In this position, the E→K mutation would elevate an external energy barrier for divalent cation entry into the binding locus, but would not increase a more distal energy barrier impeding exit downstream. In contrast, E₃Q seems most critical for divalent cation binding, as if its carboxyl group were strategically located in the middle of the binding locus. These ideas need testing further, but they embody the general principle that the inequality of the glutamate residues may be functionally advantageous (Fig. 4c, d). The outermost carboxylate might help attract a newly incoming Ca^{2+} into the binding locus, generating competition with an already bound Ca^{2+} for coordinating ligands. This process would hasten the dissociation of Ca^{2+} from the inner carboxylates and thus promote a high rate of Ca^{2+} influx. □

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HY4 gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor

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SPECIFIC responses to blue light are found throughout the biological kingdom. These responses—which in higher plants include phototropism, inhibition of hypocotyl elongation, and stomatal opening¹—are in many cases thought to be mediated by flavin-type photoreceptors². But no such blue-light photoreceptor has yet been identified or isolated, although blue-light responses in plants were reported by Darwin over a century ago³, long before the discovery of the now relatively well characterized red/far-red light photoreceptor, phytochrome⁴. Here we describe the isolation of a gene corresponding to the *HY4* locus of *Arabidopsis thaliana*. The

hy4 mutant⁵ is one of several mutants⁶ that are selectively insensitive to blue light during the blue-light-dependent inhibition of hypocotyl elongation response, which suggests that they lack an essential component of the cryptochrome-associated light-sensing pathway. The *HY4* gene, isolated by gene tagging, was shown to encode a protein with significant homology to microbial DNA photolyases. As photolyases are a rare class of flavoprotein that catalyse blue-light-dependent reactions⁷, the protein encoded by *HY4* has a structure consistent with that of a flavin-type blue-light photoreceptor.

We isolated several new mutant alleles of *HY4*. To confirm an earlier observation that these mutants are impaired in blue-light-dependent inhibition of hypocotyl elongation⁵, wild-type and *hy4* seedlings were grown under various light sources and the length of the hypocotyls measured after six days (Fig. 1); a mutant deficient in the red-light photoreceptor (phytochrome) (*hy1*)⁵ was included for comparison. All *hy4* alleles gave an impaired response to blue light compared with both wild type and *hy1*, and all alleles gave a slightly inhibited response to ultraviolet light compared with wild type. In contrast, *A. thaliana* carrying any of the *hy4* alleles responded normally to red or far-red light, unlike plants with *hy1*, which are insensitive to this region of the spectrum.

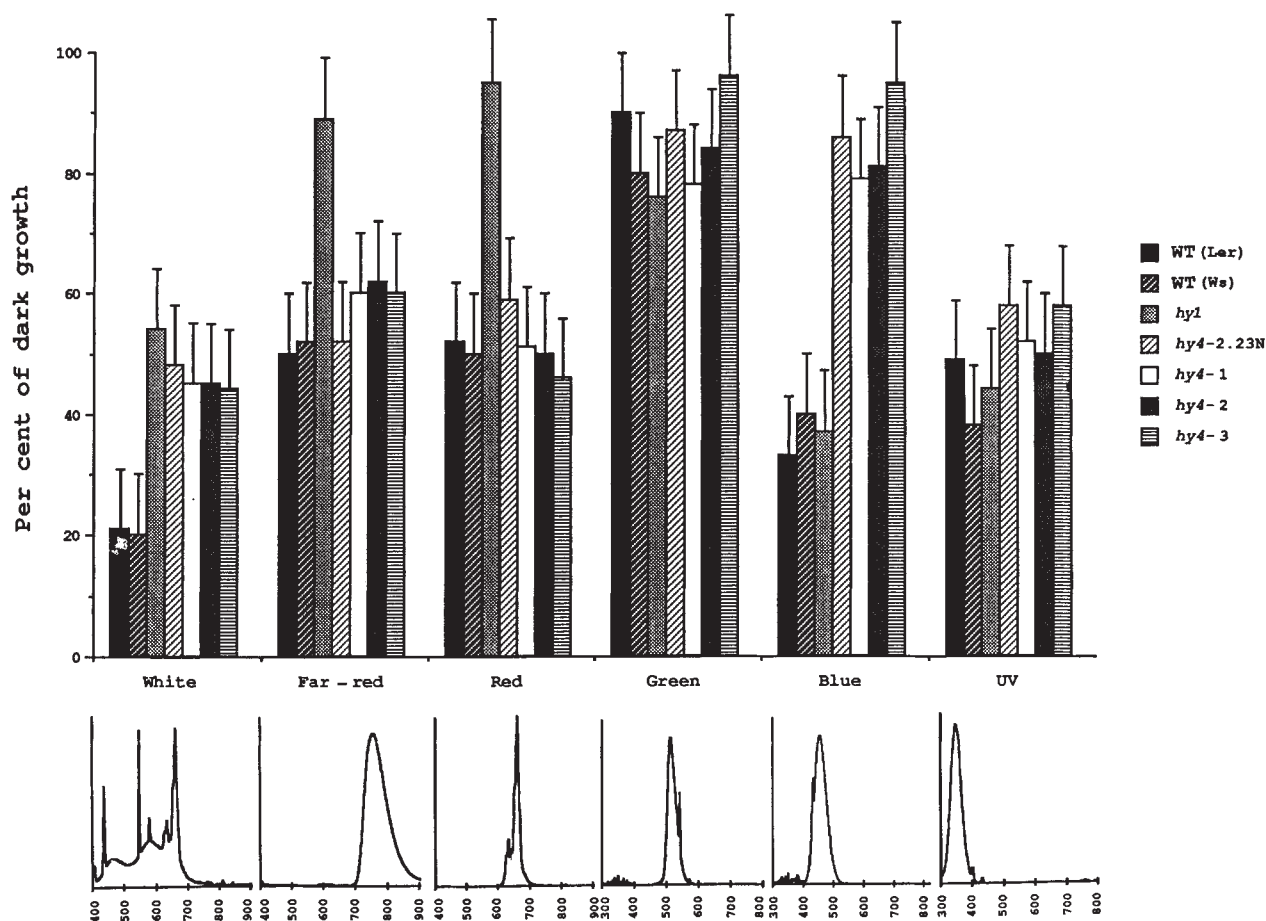


FIG. 1 Inhibition of hypocotyl elongation in alleles of *HY4*. Seeds of alleles of *HY4* (*hy4-2.23N*; *hy4-1*; *hy4-2*; *hy4-3*), *hy1* (ref. 5), and wild-type ecotypes Landsberg erecta (Ler; parental background of *hy4-2.23N*, *hy4-1*) and Ws (Wassilewskija, background of *hy4-2*, *hy4-3*) were plated on MS¹⁸ agar plates and stored for two days at 4 °C before germination. Plates were shifted to white light for 36 h to induce germination and transferred to the light condition indicated for an additional 5 days. For each treatment, hypocotyl lengths from 10 seedlings were measured and expressed as a percentage of the hypocotyl length of seedlings from a control plate that had been grown in darkness after induction of germination (hypocotyl lengths for dark-grown seedlings

were similar for all mutants and parental ecotypes). Experimental light sources were from Sylvania (Danvers, MA); the bulb type, filters and photon fluence rates ($\mu\text{mol m}^{-2} \text{s}^{-1}$) were as follows: far-red, F40/232/RS, filter FRF 700 (Westlake Plastics, Lenni, PA), fluence 17 (700–800 nm); red, F40/2364/RS, filter Red Shinkolite (Argo Plastic, Los Angeles), fluence 26; green, F40/2196/RS, filter 2092 (Polycast Technology, Stanford), fluence 25; blue, F40/246, filter 2424 (Polycast Technology), fluence 28; UV, F40 BLB, fluence 25; white, 'cool white', fluence 65. Photon fluence rates and spectra (shown here) were determined by a LabSpec VNIR 512 spectroradiometer (Analytical Spectral Devices, Boulder, CO).

We isolated a mutant allele of *HY4* (*hy4-2*) from a population of 8,000 transgenic lines of *Arabidopsis* containing random T-DNA insertions⁸. The identity of this allele was established genetically by the inability of the tagged mutant to complement the original *hy4* allele (*hy4-2.23N*)⁵. Furthermore, *hy4-2* was outcrossed to wild-type plants and 2,500 F₂ progeny were screened for cosegregation of the *hy4* phenotype with resistance to kanamycin. All of the seedlings with a *hy4* phenotype were kanamycin-resistant and therefore contained the T-DNA insertion.

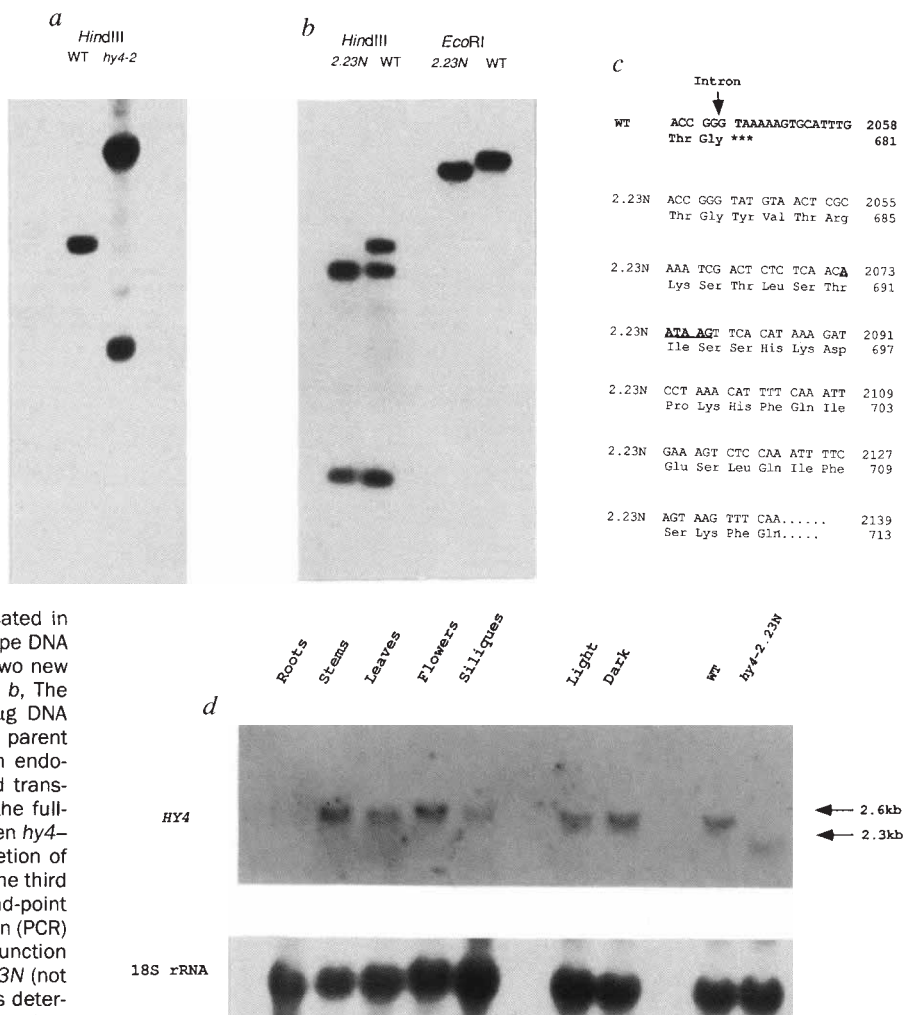
DNA flanking the site of T-DNA insertion was rescued and used to probe a Southern blot of genomic DNA from both the *hy4-2* and the wild-type parent (*A. thaliana*, ecotype Ws). As seen in Fig. 2a, the probe (containing T-DNA border as well as plant sequences) hybridized to a single band in wild-type DNA, whereas this DNA was disrupted in *hy4-2*, indicating that the plant sequences had arisen from the site of T-DNA insertion.

The cloned plant DNA immediately adjacent to the site of T-DNA integration was subsequently used to screen an *Arabidopsis* complementary DNA library⁹ and ten overlapping cDNAs were isolated. The longest of these was sequenced and found to contain an open reading frame encoding a predicted protein of 681

amino acids (*M_r* 75.8K) downstream of the first in-frame ATG (Fig. 3). The sequence of subsequently isolated genomic clones revealed the presence of three introns within the coding region, one of which occurs at the nucleotide immediately preceding the termination codon.

Confirmation that the cDNA we had cloned and sequenced corresponded to the *HY4* gene was provided by the sequence of four additional *hy4* alleles. The original *hy4* allele (*hy4-2.23N*) was generated by fast neutron irradiation⁵, and was therefore expected to result from a gross DNA rearrangement. Indeed, comparison by Southern analysis of this *hy4-2.23N* allele with the isogenic wild-type parent revealed a deletion that began within the third intron, removing the 3' intron boundary and 3' untranslated region of the cDNA but leaving the promoter and almost the entire coding sequence of the *HY4* gene intact (Fig. 2b). Owing to the presence of sequences from the unspliced intron, the coding region in *hy4-2.23N* is extended. There is no in-frame stop codon within 150 bases of the intron 5' junction (Fig. 2c), but a possible polyadenylation sequence (AATAAG) appears close to the intron 5' boundary. Northern analysis confirmed that the transcript in *hy4-2.23N* was shorter by ~300

FIG. 2 Southern and northern blot analysis of alleles of *HY4*. a, The *HY4* locus is disrupted by T-DNA in the *hy4-2* allele. Plant genomic DNA flanking the site of T-DNA insertion was cloned by standard methods¹⁹. Genomic DNA was prepared from *hy4-2* (the T-DNA-tagged mutant), digested with *Sall* and *BstEII* (leaving vector sequences intact), ligated (for recircularization), electroporated into *E. coli*, and then plated on ampicillin-selection medium. Colonies, arising as a result of both the bacterial antibiotic-resistance genes and the origin of replication conferred by the T-DNA, were screened for the presence of plant DNA flanking the site of T-DNA integration. Two such colonies were identified, each containing ~15 kb of plant genomic sequence. DNA (5 µg) from *hy4-2* and from the wild-type parental Ws strain (WT) was digested with *HindIII*, resolved on a 0.7% agarose gel and blotted to nitrocellulose. Blots were probed with a fragment of rescued DNA containing left-border vector sequences as well as plant genomic DNA downstream of the site of T-DNA insertion (indicated in Fig. 3). The hybridizing band present in the wild-type DNA is absent in the mutant, where it is replaced by two new hybridizing bands due to the insertion of T-DNA. b, The *hy4-2.23N* allele contains a small deletion. 5 µg DNA from *hy4-2.23N* and the wild-type isogenic parent (*Landsberg erecta*) were digested with restriction endonucleases, resolved on a 0.7% agarose gel, and transferred to nitrocellulose. Blots were probed with the full-length *HY4* cDNA. The difference in pattern between *hy4-2.23N* and wild-type represents an apparent deletion of ~2 kb in the mutant. c, Sequence extending into the third intron of *HY4*. Further analysis of the deletion end-point by Southern blotting and polymerase chain reaction (PCR) product analysis revealed that the 3' intron splice junction and 3' untranslated region are absent in *hy4-2.23N* (not shown). The sequence of the unspliced intron was determined from genomic sequence of *hy4-2.23N*, extending the open reading frame and encoding a putative polyadenylation site (underlined). d, Transcript analysis of *HY4*. RNA was prepared from roots, stems, leaves, flowers and immature siliques of 5-week-old plants by standard methods²⁰. The dark-adaptation experiment involved 3-week-old leaf tissue from plants either grown in continuous white light ('Light') or placed in the dark for two days before sampling ('Dark'); light-grown and dark-grown 5-day-old seedlings contained similar amounts of *HY4* transcript which were not significantly



different from levels in the samples shown here (data not shown). Wild-type (*Landsberg erecta*) and mutant (*hy4-2.23N*) RNA was from 3-week-old leaf tissue. RNA (40 µg) was resolved on a 1% formaldehyde agarose gel and transferred to nitrocellulose. The blot was probed under high stringency conditions with radiolabelled *HY4* cDNA and exposed for 4 days to X-ray film for autoradiography. The 18S ribosomal RNA for each sample is shown for comparison.

base pairs (bp) than the wild-type *HY4* transcript (Fig. 2d), suggesting that the spurious intron polyadenylation signal may indeed be used. Moreover, transcript levels were reduced, possibly as a result of inefficient processing or greater instability of messenger RNA.

Two mutant alleles of *HY4* generated by ethyl methane sulphonate (EMS) mutagenesis (*hy4-1* and *hy4-4*), and an untagged allele (*hy4-3*) isolated from the T-DNA-tagged lines, were also characterized. Amino-acid substitutions (Gly 340→Glu and Gly 337→Asp) were identified respectively in the *hy4-1* and *hy4-4* EMS alleles, and a 5-bp deletion (nucleotides 1,636–1,640) which led to a frameshift and premature termination was identified in *hy4-3* (Figs 3 and 4b, c).

The amino-acid sequence of *HY4* shows striking sequence homology with the microbial DNA photolyases, a class of flavoproteins that catalyse the light-dependent repair of pyrimidine dimers in DNA damaged by ultraviolet light⁷. Optimal alignment of the sequence of *HY4* with those of seven characterized microbial photolyases (Fig. 4a) revealed a sequence identity as high as 30% over a stretch of 500 amino acids; regions of homology as high as 70% (*Escherichia coli* photolyase, amino acids 5–45) or even 80% (amino acids 330–371) were identified. Interestingly, the homology was highest in regions known to be involved in photolyase chromophore binding¹⁰; the carboxy-terminal domain binds a reduced flavin (FADH₂), whereas the amino-terminal domain binds either a pterin (in the so-called short-wavelength photolyases having their absorption maximum at 380 nm) or a deazaflavin derivative (in the long-wavelength photolyases, absorption maximum ~435 nm). The functional

significance of this homology is indicated by the fact that two of the mutations we have identified in alleles of *hy4* (*hy4-1* and *hy4-4*) are amino-acid substitutions in a region of exceptional sequence conservation in photolyases (Fig. 4a). Moreover, disruption of the homologous region of the *E. coli* enzyme by a linker insertion mutation inactivates the enzyme¹¹. A tryptophan residue (conserved in all seven photolyases) that has been implicated¹² in specific recognition of the pyrimidine-dimer substrate (*E. coli* Trp 277) is not conserved in *HY4*.

Analysis of the amino-terminal region of *HY4* indicates more sequence relatedness to the long-wavelength class of photolyases, which possess a conserved Phe (Phe 34 in *S. griseus*) and a conserved sequence (Pro, His/Ala, Leu, His/Lys, Phe; residues 236–240 in *S. griseus*) indicative of long-wavelength-type photolyases^{13,14}. *HY4* might therefore be expected to bind a deazaflavin derivative as a second chromophore, which would account for the relative spectral sensitivity determined for *HY4* (Fig. 1). But the *HY4* chromophores have still to be precisely identified. In contrast to our observations for *HY4*, *Arabidopsis* photolyase activity has been reported to be primarily of the short-wavelength type¹⁵.

HY4 also shows significant sequence relatedness (30% identity and 45% relatedness over a stretch of 86 amino acids) to rat smooth-muscle tropomyosin A¹⁶ (Fig. 4b). This region of similarity of *HY4* to tropomyosin is limited to the C-terminal third of *HY4*, which is distinct from the region of homology to photolyases. Two of the mutant alleles of *HY4* that we have characterized result from disruptions within this C-terminal domain (Figs 3 and 4c), one of these being the T-DNA insertion

-147	TCAAAAATCTTTTCTTTTCTGTTCTTCTCTCGAGAGATAAGTACCAAGGTTTCATTCTCGAAATAGTTTGAATAAA	
-61	AAAGTAATTTTATGTGATTATGCCAAGAAAGTTTATGTTTTTATGTTTCTGAGAG	
	MetSerGlySerValSerGlyCysGlySer GlyGlyCysSerIleValTrpPheArgArg	20
61	GATCTTAGGTTGAAGATAATCCAGCTTTA GCAGCAGCAGTAAGAGCTGGTCCAGTGATT	
	AspLeuArgValGluAspAsnProAlaLeu AlaAlaAlaValArgAlaGlyProValIle	60
181	TGGTGGCTCAAGAACGTTTGGCTCAGCTT GATTCTCTCTTAGAAGCTTTGGTACTTGT	
	TrpTrpLeuLysAsnSerLeuAlaGlnLeu AspSerSerLeuArgSerLeuGlyThrCys	100
301	GGTGCTCTCAGATCTCTTCAACCATTTG TATGATCCATTGCTTTGGTGGGTGATC	
	GlyAlaSerGlnIlePhePheAsnHisLeu TyrAspProLeuSerLeuValArgAspHis	140
421	CTTTATGACCATGGGAAGTGACTGATGAA TTAGGCGCTCTTCTCTATGTTTGGTGGC	
	LeuGlyArgProPheSerMetPheAlaAla PheTrpGluArgCysLeuSerMetProTyr	180
541	AAGATCATTTACGGGATGTGTCTAAATGT GTTGGGATCATCTGGTCTTGAAGTAC	
	LysIleIleSerGlyAspValSerLysCys ValAlaAspProLeuValPheGluAspAsp	220
661	GATTAAGCTCTCAACGTTTAAACCGGT CCATTGCTGAATCTCTAAGAACGCTAG	
	AspLysAlaLeuThrThrPheIleAsnGly ProLeuLeuGluTyrSerLysAsnArgArg	260
781	GTGAGAAAGTTTTCATCTCTTGGATC AAACAGGTGGCTGGGCAACGAGAAAC	
	ValArgLysValPheHisIleuValArgIle LysGlnValAlaTrpAlaAsnGluLysAsn	300
901	AGGTACATTAAGTTTAAACCATCATATCC CATGAAGACCATCTCTTGGCCATCTAAG	
	ArgTyrIleSerPheAsnHisProTyrSer HisGluArgProLeuLeuGlyHisLeuLys	340
1021	TATCGGTGGTGGTGGGATGAGAGAG TTATGGGTACTGGTGGTGCATGATCGC	
	ValArgLysValPheHisIleuValArgGlu LeuTrpAlaThrGlyTrpLeuHisAspArg	380
1141	ATGAAGTATTTCTGGGACACCTCTTGAT GCGGATTAGAAGCGATGCTCTTGTTGG	
	MetLysTyrPheTrpAspThrLeuLeuAsp AlaAspLeuGluSerAspAlaLeuGlyTyr	420
1261	TTTGAAGGTTCAAGTTTGTATCCAAATGGT GAATACGTAAGCGGATGGTCTCGTACCTC	
	PheGluGlyTyrLysPheAspProAsnGly GluTyrValArgArgTrpLeuProGluLeu	460
1381	GCTGCTGATCGAGCTTGGATCAAACTAT CCTCTACCAATTTGTGATTAGCAGGAACA	
	AlaAlaGlyIleGluLeuGlySerAsnTyr ProLeuProIleValGlyLeuAspGluAla	500
1501	GCAATGAGACCGGATCCGAGAGAGACTT GGAGATTCTGCTGAGTGAGAGAGCTCCT	
	AlaIleGluAsnGlySerGluGlyLeu GlyAspSerAlaGluValGluGluAlaPro	540
1621	ACGAGATATGAGGATCGAGGTTCCAAAGC ATATCTCTCTTCTTGATCAGACCTGAGAA	
	ArgArgTyrGluAspGlnMetValProSer ILeThrSerSerLeuAlaGluProGluGlu	580
1741	AGGAACATGGTTAAACCAACCAAGCTCAG CAGCGGAGAGCAGAACCGGCTTCAACCAA	
	ArgAsnMetValAlaAsnThrAsnGlnAlaGln GlnArgArgAlaGluProAlaSerAsnGln	620
1861	ACAGCGGATCTTCCAGCAGCGGAAGGAGA GAAAGAGCGGAGGATAGTCCCGAGTGG	
	ThrAlaGluSerSerSerSerGlyArgArg GluArgSerGlyGlyIleValProGluTrp	660
1981	ACGCTAGCTACTTGCAGATCACCATTGAA ATACTGAATCGAGCGGCTTCAACAACT	
	ThrSerSerTyrLeuGlnAsnHisIleGlu IleLeuAsnTrpArgArgSerGlnThr	700
2101	GGTGAATCTGGTGGATCTGAACCGAGTACATTTGGTACGGTTTAAATGTAATTCGGTTAT	
2225	TCATCATGTATAATACACTGATAGTAACTGCTGTGGTATTAGACCGAGTCTCATCTTGTGGCTTCAAGTTT	

FIG. 3 DNA sequence of the *HY4* cDNA and the encoded protein. The nucleotide sequence of the longest of 10 identical cDNAs, isolated from a cDNA library of ecotype Columbia, was determined mechanically by the Sanger dideoxy sequencing protocol²¹ on an Applied Biosystems 370A automated DNA sequencer. The 5' and 3' ends of three additional cDNAs were sequenced to confirm translation initiation and termination sites. The positions of three introns were determined by sequencing of a genomic clone. Mutant alleles of *HY4* were also characterized. PCR amplification of the coding sequence from genomic DNA was carried out in at least 3 independent reactions for each mutant. These PCR

amplification products were determined directly and mutations within the cDNA coding sequence identified. As the identical nucleotide substitution or deletion was found in each of the three independently generated PCR products from each allele, the possibility of PCR artefacts generating these mutations was eliminated. The location and nature of the mutations in different *hy4* alleles are given: *hy4-1* and *hy4-4* are point mutations and the nature of the nucleotide and corresponding amino-acid changes are given; the T-DNA insertion in *hy4-2* is marked and the 5-bp deletion of *hy4-3* is underlined.

possibility that the *HY4* gene encodes the apoprotein of a blue-light photoreceptor. The *hy4* mutant phenotype specifically impairs blue-light responsiveness in *Arabidopsis*. The *HY4* gene encodes a protein with significant homology to a very rare class of flavoprotein that catalyses blue-light-dependent reactions. This homology is shown to be functionally significant by our identification of point mutations in conserved domains that result in inactivation of both *HY4* and photolyases. The action spectrum of blue-light inhibition of hypocotyl elongation matches the absorption spectrum of the homologous long-wavelength class of photolyases. If the *HY4* protein were to retain some photolyase activity, such activity would not account for the *hy4* elongated hypocotyl phenotype, which occurs under visible light in the absence of pyrimidine-dimer formation.

The hydrophilicity profile of *HY4* indicates no obvious membrane-spanning domain (data not shown) and the protein would appear to be soluble (C. Lin, unpublished results), so the blue-light-dependent phosphorylation activity in isolated *Arabidopsis* membranes¹⁷ is unlikely to involve *HY4*. The likelihood that there may be several related blue-light photoreceptors, each with a distinct function, as in the phytochromes⁴, is in agreement with an observation that *HY4* forms part of a small gene family (J. Chan, unpublished results). Furthermore, our results could help provide insight into the structure in other organisms of blue-light photoreceptors that may also be related to photolyases. □

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Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon- γ signal transduction pathway

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INTERFERONS (IFNs) α/β (type I) and γ (type II) bind to distinct cell surface receptors¹, inducing transcription of overlapping sets of genes by intracellular pathways that have recently attracted much attention^{2,3}. Previous studies using cell lines selected for their inability to respond to IFN- α (ref. 4) have shown that the protein kinase Tyk2 plays a central role in the IFN α/β response⁵. Here we report the isolation of the cell line γ 1A, selected for its inability to express IFN- γ -inducible cell-surface markers, that is deficient in all aspects of the IFN- γ response tested, but responds normally to IFNs α and β . The mutant cells can be complemented by the expression of another member of the JAK family of protein tyrosine kinases, JAK2 (refs 6–9). Unlike IFNs α and β , IFN- γ induces rapid tyrosine phosphorylation of JAK2 in wild-type cells, and

JAK2 immunoprecipitates from these cells show tyrosine kinase activity. These responses are absent in γ 1A cells. JAK2 is therefore required for the response to IFN- γ but not to IFNs α and β .

The 9-27 gene promoter is inducible by IFN- γ as well as IFN- α and - β ¹⁰. Significant constitutive expression from this promoter precluded a drug-selection protocol. Accordingly, a clone of cells (2C4) expressing the simple cell-surface marker CD2 (ref. 11) (normally expressed only on T cells) under the control of the 9-27 promoter was derived and the fluorescence-activated cell sorter (FACS) used to screen for loss or gain of IFN- γ inducibility. IFN-inducible expression of endogenous class I and II HLAs was also monitored. In 2C4 cells there was good induction of all three antigens by IFN- γ and of CD2 and class I by IFN- α (panels 1–3 in Fig. 1). Mutant γ 1A was isolated by mutagenesis of 2C4 and several rounds of sorting. To enhance the isolation of *trans* rather than *cis* mutants and of mutants in the primary rather than secondary IFN- γ response pathways, sorting was carried out on both CD2 and class I antigens. Mutant γ 1A is defective in the response to IFN- γ but not to IFN- α (panels 4–6 in Fig. 1) or IFN- β (data not shown). Transfection of this mutant with a murine JAK2 expression construct⁹, however, restored the IFN- γ response of all three cell-surface markers in an enriched population (panels 7–9) and clones (for example, panels 10–12) of transfectants (Fig. 1). Transfection of γ 1A with murine JAK1 or with human Tyk2 was without effect. In contrast, JAK1 complements mutants in complementation group U4 (ref. 3) and, as previously shown⁵, Tyk2 complements mutants in complementation group U1. In the inverse experiments, JAK2 did not complement mutants in complementation group U1 or U4 (D.W., M.M. and I.M.K., data not shown).

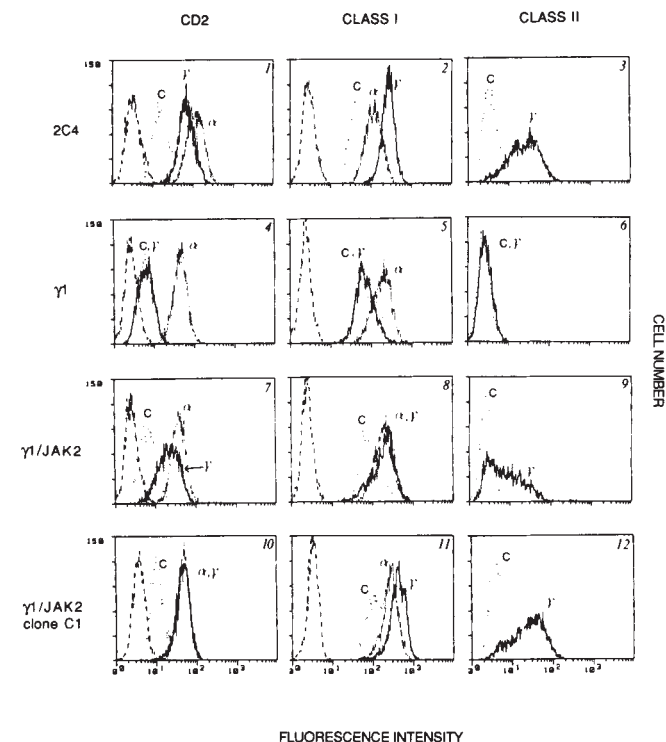
The expression of a range of IFN- γ -inducible messenger RNAs was compared in 2C4 and γ 1A cells and in γ 1A/JAK2 transfectants by RNase protection (Fig. 2). For all eight IFN-inducible mRNAs tested (Fig. 2a and b), the IFN- α response (minimal for IRF1 and GBP; Fig. 2b) was the same for 2C4 (lanes 1–3), mutant γ 1A (lanes 7–9) and the γ 1A/JAK2 transfectants (lanes 13–15), whereas for IFN- γ the response in 2C4 (lanes 4–6) was lost in γ 1A (lanes 10–12) but was restored for all mRNAs by JAK2 (lanes 16–18). The IFN- γ dose-response curves for the wild-type 2C4 and γ 1A/JAK2 transfectants were essentially identical: a clear response was seen at 10 IU ml⁻¹ and

an approaching maximal response at 100 IU ml^{-1} (D.W. and I.M.K., data not shown).

The p91 component of the IFN- α/β -inducible transcription factor ISGF3 is rapidly phosphorylated on tyrosine in response to either type of IFN^{12,13} and is required for the IFN- γ response

of a wide spectrum of genes¹⁴. It appears to correspond to the γ activation factor (GAF) implicated in the activation of several genes in response to IFN- γ through a common DNA motif¹⁵⁻¹⁹. Phosphorylation of p91 in response to IFN- γ was therefore assayed in wild-type and mutant $\gamma 1A$ cells and in $\gamma 1A$ /

FIG. 1 Cell-surface expression of transfected CD2 and endogenous class I and II HLAs in response to IFNs α or γ on wild-type 2C4 (panels 1–3), mutant $\gamma 1A$ (panels 4–6) cells and mutant $\gamma 1A$ cells stably transfected with a murine JAK2 cDNA expression construct (panels 7–12). Data for an enriched population (panels 7–9) and a clone (panels 10–12) of $\gamma 1A$ /JAK2 transfectants are presented. FACSCAN (Becton Dickinson) analysis (3,000 data points, consort 30) are shown. Dashed line, nonspecific antibody to keyhole limpet haemocyanin (X39; Becton Dickinson) with or without IFN treatment; solid and dotted lines control (C), and IFN- α or - γ treated, as indicated. Cells were plated at 5×10^5 per 10-cm dish and treated the next day with 10^3 IU per ml of a highly purified mixture of α -IFNs (Wellferon, 1.5×10^6 IU per mg protein; Wellcome Research) or recombinant human IFN- γ (2×10^7 IU per mg protein; gift from G. Adolf). Cells (10^6) were stained for 30 min at 0°C with R-phycoerythrin-conjugated murine monoclonal antibody to human CD2 (Dako-CD2 MT910; Denmark) or HLA DRA (clone L243, Becton Dickinson), or FITC-conjugated murine monoclonal antibody to human HLA ABC (shared determinant, clone W6/32; Seralab, UK) and fixed in 1% paraformaldehyde. Clone 2C4 was derived by stable co-transfection of human HT1080 cells with pDW9-27CD2 and pTKNeo and FACSCAN analysis of G418-resistant clones. pDW9-27CD2 is a modification of PJ3omega²⁵ in which the SV40 promoter was replaced by the 1.8-kb *HindIII* to *BspMII* promoter fragment of the 9–27 gene¹⁰ and which carries a full-length CD2 cDNA¹¹ in the *EcoRI* site of the polylinker. Mutagenesis (five rounds) with ICR191 was done as previously described⁴. Cells not responsive to IFN- γ were 'selected' using a FACSTAR-Plus cell sorter (Becton Dickinson). 5×10^7 mutagenized cells were treated with 500 IU per ml of recombinant human IFN- γ for 48 h, resuspended and stained with phycoerythrin-conjugated antibody to CD2 and FITC-conjugated antibody to HLA class I (above) and sorted immediately. The bottom 5% of fluorescing cells were collected. After six rounds of sorting, clone $\gamma 1A$ was isolated by limiting dilution of the enriched population. It showed a novel IFN- γ $\alpha^+ \beta^+$ phenotype. The phenotype was stable on continuous culture for at least three months. Mutant $\gamma 1A$ was complemented by transfection with a full-length cDNA of murine JAK2 downstream of the cytomegalovirus promoter in pRK5 in the presence of a puromycin-selectable marker plasmid. The puromycin-resistant population of stable transfectants were treated with recombi-



nant IFN- γ , FACS-sorted and the top 7% of responder cells were collected and analysed (panels 7–9). Clones of $\gamma 1A$ /JAK2 transfectant cells, obtained by limiting dilution of the enriched population, were also analysed, when the IFN- γ response was found to be fully restored (panels 10–12).

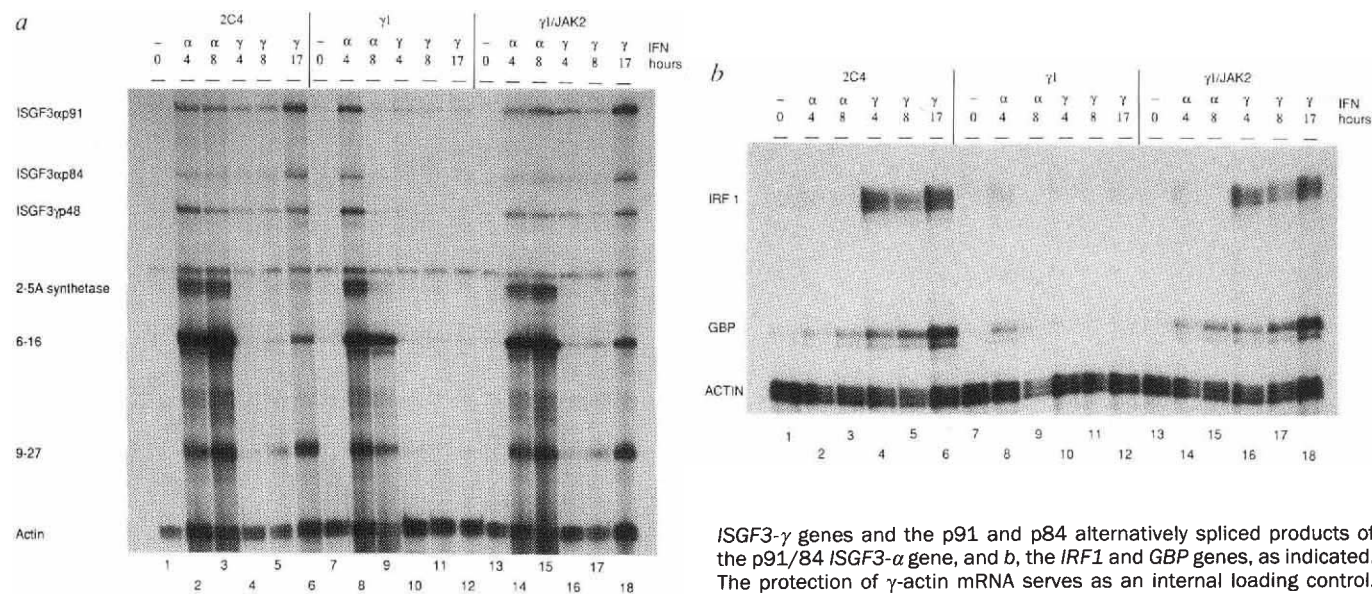


FIG. 2 IFN-inducible gene expression in wild-type 2C4, mutant $\gamma 1A$ and mutant $\gamma 1A$ /JAK2 transfectant cells. mRNA expression in response to IFNs- α or - γ was monitored by RNase protection using probes to detect the IFN-inducible mRNAs of a, the 9-27, 6-16, 2-5A synthetase and

ISGF3- γ genes and the p91 and p84 alternatively spliced products of the p91/84 ISGF3- α gene, and b, the *IRF1* and *GBP* genes, as indicated. The protection of γ -actin mRNA serves as an internal loading control. IFN treatment was as described for Fig. 1 but for the times indicated. Cytoplasmic RNA was prepared from monolayer cells by NP-40 lysis and phenol/chloroform extraction. RNase protection was with RNA probes labelled with ^{32}P -UTP to $2\text{--}5 \times 10^8$ c.p.m. per μg of input DNA¹⁴. $1\text{--}3 \times 10^5$ c.p.m. of each probe and $10 \mu\text{g}$ RNA were used in each assay.

JAK2 transfectants (Fig. 3a and b). Incorporation of $^{32}\text{P}_i$ into p91 occurs rapidly in parental 2C4 cells. There was no phosphorylation in mutant $\gamma 1A$, but it was restored in $\gamma 1A$ complemented by JAK2 (Fig. 3a). Similar results were obtained when phosphorylation was monitored with antibodies against phosphotyrosine (Fig. 3b, top panel). Normal levels of p91 are present (Fig. 3b, bottom panel), and, interestingly, phosphorylation of the p91 and p113 components of ISGF3- α by IFN- α is

normal in the mutant cells (Fig. 3c). (Levels of the p84 component of ISGF3 α are usually lower than p91, and phosphorylation in response to IFN- α or - γ is often difficult to detect^{12,13}. This was the case throughout the experiments described here.) In addition, the mutant $\gamma 1A$ cells are not complementable by a functional p91 expression construct (D.W., C.S., J. E. Darnell Jr, G.R.S. and I.M.K., unpublished results). Taken together, the data indicate that the defect in $\gamma 1A$ cells is upstream of p91.

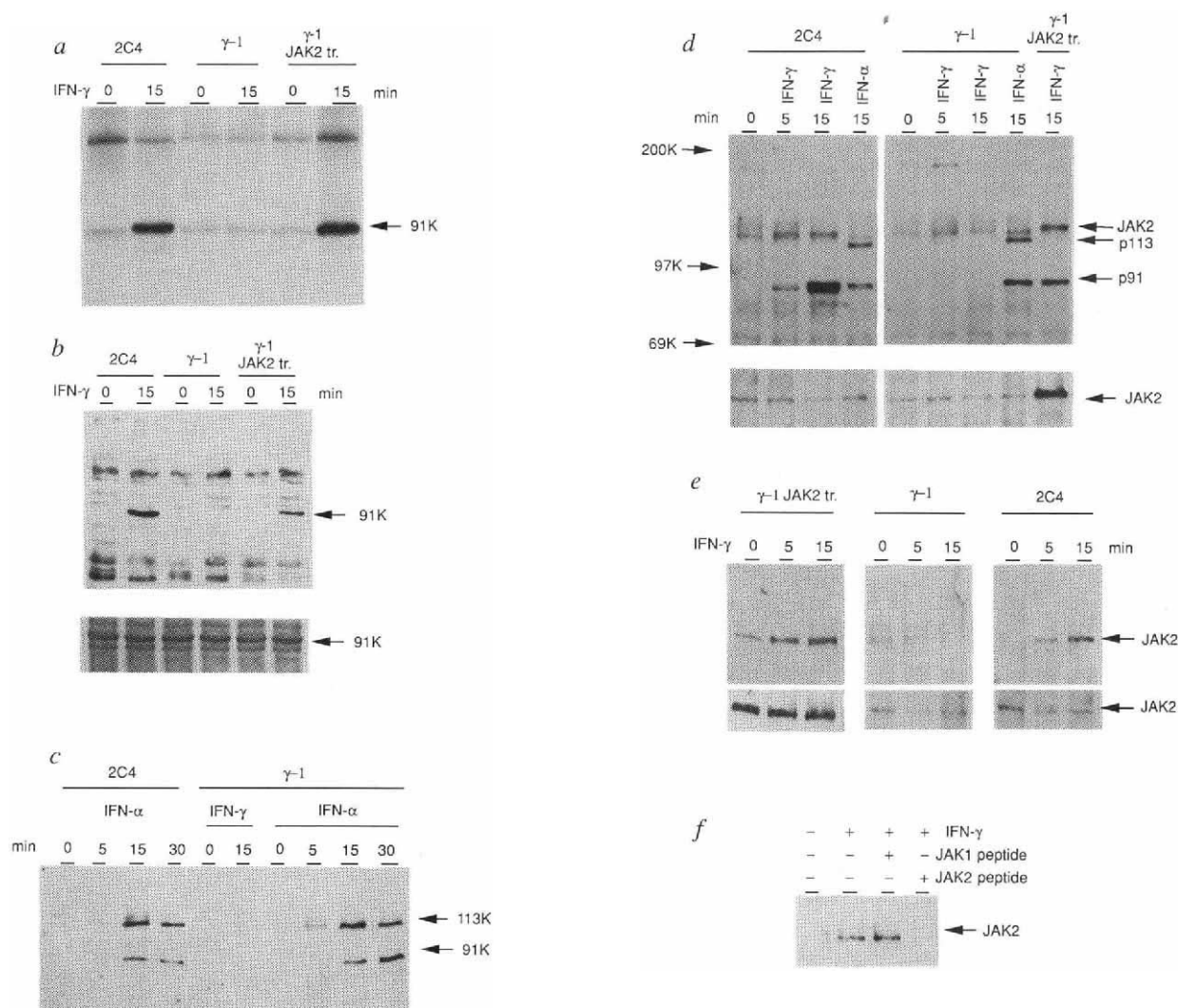


FIG. 3 Tyrosine phosphorylation of p91 (a–c), p113 (c) and JAK2 (d–f) in response to IFNs- γ or - α in wild-type and mutant $\gamma 1A$ cells and $\gamma 1A$ /JAK2 transfectants (tr). Phosphorylation of p91 in response to IFN- γ was monitored by a, incorporation of $^{32}\text{P}_i$, or b (top) and c, western transfer with antibody to phosphotyrosine. b (bottom panel), After stripping, p91 protein levels were monitored on the same (top panel) transfer with antibody to p91. c, Tyrosine phosphorylation of the p91 and p113 components of ISGF3 in response to IFN- α . c–f, Phosphorylation of JAK2. d (top), Extracts from control and IFN- γ treated cells were immunoprecipitated with a mixture of antibodies to JAK2 and, as controls, to p91/84. Extracts from cells treated with IFN- α were precipitated with a mixture of antibodies to JAK2 and p113 (the latter co-precipitates p91 in IFN- α activated ISGF3). Phosphorylation on tyrosine was monitored as in b, (top panel). Note: the background band that migrates close to the position of JAK2 in the $\gamma 1A$ lanes has not been consistently observed. d (lower panel), to detect JAK2 protein, the same blot (top panel) was stripped and reprobed with antibody to JAK2. e, Extracts were prepared and treated as in d, except that in the immune precipitation antibodies to p91/84 and p113 were not included with the antibody to JAK2 and control extracts were included from the $\gamma 1A$ /JAK2 transfectants to show the background level of phosphorylation of JAK2 observed in extracts

from these cells. f, Specificity of the immune precipitation of JAK2. Extracts from 2C4 cells treated with IFN- γ for 15 min were immunoprecipitated with antibody to JAK2 in the presence or absence, as indicated, of 0.1 mg ml⁻¹ of the JAK2 peptide against which the JAK2 antibody was raised⁹ or an unrelated JAK1 peptide. Immunoprecipitates were analysed for phosphotyrosine as described. Cells were grown and treated with 10³ IU per ml of highly purified IFN- γ or - α for the times indicated as for Fig. 1. For incorporation of $^{32}\text{P}_i$ (a), 5 × 10⁶ cells were incubated with 0.5 mCi ml⁻¹ $^{32}\text{P}_i$ at 37 °C for 1 h. The cells were then lysed and analysed as below. Immunoprecipitations were from pre-cleared whole-cell extracts (10⁷ cells) using appropriate antisera and protein A-Sepharose (Pharmacia) as described^{12,13}. Analysis was by SDS-PAGE and western transfer with, for phosphotyrosine, a mixture of PY20 (ICN) and 4G10 (UBI) antiphosphotyrosine antibodies and, after stripping in 0.1 M glycine buffer, pH 2.5, for 30 min and neutralization in 0.1 M Tris-HCl, pH 8.0, with antibodies against p91 (refs 12, 13) p113 (refs 12, 13) or JAK2 (ref. 9), as appropriate, to assay the individual proteins. In the western transfers, detection was by ECL (Amersham, UK), except for the upper panel in b, which was stained with diaminobenzidine (Sigma).

Tyrosine phosphorylation and activation of JAK2 occurs in response to a variety of cytokines^{20, 22}. The phosphorylation of JAK2 in response to IFN- γ in parental and mutant cells was examined by immunoprecipitation with specific antibody, followed by western transfer analysis of the immune precipitates with antibody against phosphotyrosine. The antipeptide serum used was against a peptide unique to JAK2 relative to JAK1 or Tyk2 (refs 9, 20). JAK2 is rapidly phosphorylated on tyrosine in response to IFN- γ in wild-type but not in mutant γ 1A cells (Fig. 3d and e). No such phosphorylation of JAK2 was observed in response to IFN- α (Fig. 3d, top panel) under conditions identical to those under which phosphorylation of Tyk2 by IFN- α is readily detected (S. Holland, S. Pellegrini, M.M., D.G., G.R.S. and I.M.K., data not shown). Tyrosine phosphorylation of p91 in response to IFN- γ and of p91 and p113 in response to IFN- α were monitored in parallel as internal controls, both for IFN activity and detection of phosphotyrosine (Fig. 3d, top panel). On reprobing the same transfer with antibody to JAK2, comparable levels of JAK2 protein were detected in wild-type and mutant cells (Fig. 3d and e, lower panels). The defect in γ 1A is therefore in the phosphorylation or function rather than in the production of JAK2. Consistent with specific phosphorylation of JAK2, phosphorylated protein was not recovered when the immune precipitation was carried out in the presence of the appropriate competing peptide (Fig. 3f). In γ 1A/JAK2 transfectants, there is a high 'background' level of tyrosine phosphorylation of the overexpressed exogenous murine JAK2 even without IFN- γ treatment (Fig. 3e, top panel). The site(s) of, and the basis for, this constitutive phosphorylation, which is not observed in *in vitro* kinase assays (Fig. 4), are not known. Against this background, a variable increase in tyrosine phosphorylation of JAK2 is seen in response to IFN- γ in the complemented cells (see Fig. 3e, top panel, for example).

Activation of protein tyrosine kinases in response to growth factors classically results in kinase activity which can be detected in an immune precipitate of the activated enzyme. JAK2 activ-

ated in response to IL3 (ref. 9) and erythropoietin²⁰ shows similar *in vitro* kinase activity. This is also the case for JAK2 in response to IFN- γ (Fig. 4). IFN- γ -dependent kinase activity was observed on assay of JAK2 immunoprecipitates from parental 2C4 or mutant γ 1A/JAK2 transfectant cells (Fig. 4a). No such activity was observed in response to IFN- α (Fig. 4a) or when the immunoprecipitates were prepared from mutant γ 1A cells (Fig. 4b) or from wild-type cells in the presence of competing JAK2 peptide (data not shown). Phosphorylation of JAK2 is not restricted to human HT1080 derived cells. It is also seen in response to IFN- γ , but not IFN- α , in other human and a variety of mouse cell lines. As an example, IFN- γ -dependent phosphorylation of JAK2 on tyrosine in immunoprecipitates from mouse L-cells is shown in Fig. 4c. Neither is activity restricted to auto-phosphorylation: immunoprecipitates of JAK2 from IFN- γ -treated cells also phosphorylate the synthetic substrate poly(Glu-Tyr) 4:1 (from Sigma; D.G. and I.M.K., unpublished result).

In mutant U1A cells the defect in Tyk2 results in the loss of high-affinity binding for an IFN- α receptor. Essentially identical binding of IFN- γ was, however, reproducibly observed with parental 2C4 and mutant γ 1A cells (D.W. and I.M.K., data not shown). It will be interesting to determine whether this difference between U1A and γ 1A reflects the absence of Tyk2 but not JAK2 protein, respectively, in the two mutants, or a more fundamental difference in the presumptive interaction of the two kinases with their receptor complexes. The JAK2 protein, like Tyk2 (S. Pellegrini, personal communication) does not appear to be significantly induced in response to IFNs γ or α in wild-type cells (D.G. and I.M.K., data not shown).

The precise site of the defect in γ 1A cells is not known. A minor mutation in JAK2 may be responsible, but the defect could also be in another component which is rendered unable to interact with normal levels of JAK2 but is rescued by higher levels of transfected JAK2 (Fig. 3d and e). Irrespective of the nature of the defect, the properties of the mutant cells clearly indicate a role for JAK2 in the primary response to IFN- γ . JAK2

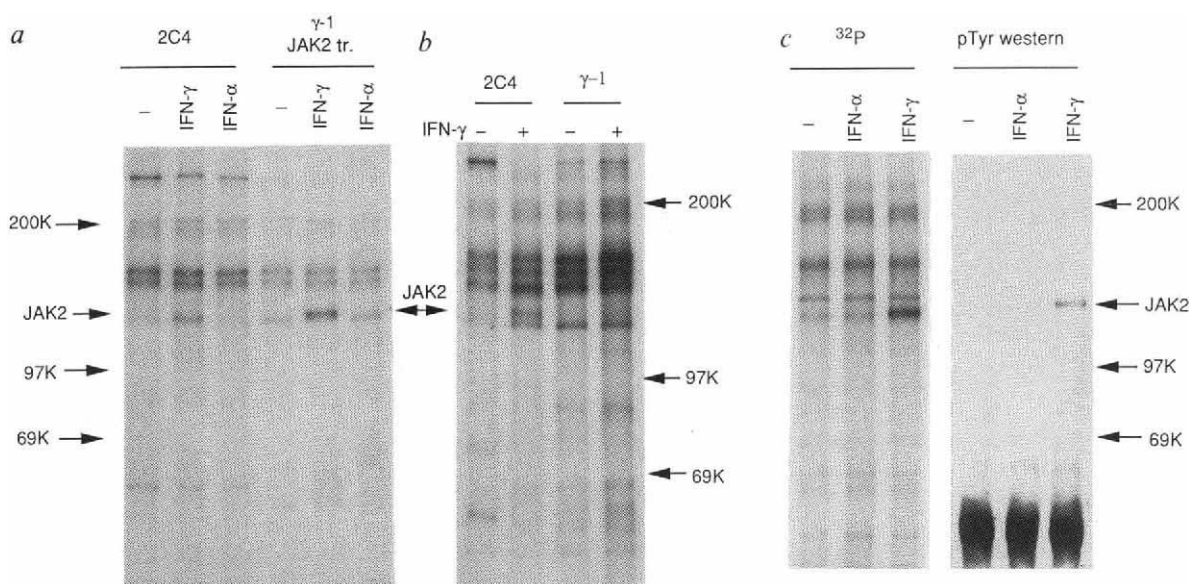


FIG. 4 *In vitro* kinase assays. IFN-dependent phosphorylation of JAK2 was assayed in immunoprecipitates from a, wild-type (2C4) and mutant γ 1A/JAK2 transfectant cells; b, wild-type (2C4) and mutant γ 1A cells, and c, mouse L-cells. Treatment with IFN- γ or - α (500 IU ml⁻¹), as indicated, was for 15 min. Immune precipitates on protein A-Sepharose (Pharmacia) were washed in 50 mM NaCl, 5 mM MgCl₂, 5 mM MnCl₂, 0.1 mM Na₃VO₄, 10 mM HEPES, pH 7.4, and incubated in the same buffer containing 0.25 mCi ml⁻¹ [³²P- γ]ATP for 30 min at room

temperature^{12,13}. After extensive washing, proteins were eluted in sample buffer and analysed by SDS-PAGE. Detection was by autoradiography or, in the last three lanes (c), by western transfer for phosphotyrosine as in Fig. 3. Growth and IFN treatment of human cells was as for Fig. 1. Growth and IFN treatment of mouse L-cells was similar, but with recombinant murine IFN- γ (1–2 $\times$ 10⁷ IU per mg protein; gift from G. Adolf or a recombinant human IFN- α A/D (Bg1) hybrid that is highly active on mouse cells (2 $\times$ 10⁸ IU per mg protein; from S. Pestka).

and JAK1 are thus required for the IFN- γ response³, and Tyk2 (ref. 5) and JAK1 (ref. 3) are required for the IFN- α and - β responses.

Two regions have been identified as being of particular importance in the cytoplasmic domain of the IFN-binding subunit of the IFN- γ receptor: a membrane proximal region and a distal sequence, including an essential tyrosine^{26,27}. Phosphorylation of this tyrosine may involve JAK2. A number of other cytokines induce activation of JAK2 (refs 20–22). Erythropoietin and interleukin-3 do not induce tyrosine phosphorylation of ISGF3 p91, however, indicating that the receptor/kinase complex confers specificity on the response (ref. 23, and C.S., B.A.W. and J.N.I., unpublished results). Cell-free p91 activation in response to IFN- γ occurs in a membrane complex²⁴ (D.G. and I.M.K., unpublished results). Specificity may therefore be mediated at least in part by direct interaction between the receptor/kinase complexes and different specific transcription factors³. □

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BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription

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SEQUENCE-SPECIFIC DNA binding activators of gene transcription may be assisted by SWI2(SNF2)^{1,2}, which contains a DNA-dependent ATPase domain³. We have isolated a human complementary DNA encoding a 205K nuclear protein, BRG1, that contains extensive homology to SWI2 and *Drosophila brahma*^{4,5}. We report here that a SWI2/BRG1 chimera with the DNA-dependent ATPase domain replaced by corresponding human sequence restored normal mitotic growth and capacity for transcriptional activation to *swi2*[−] yeast cells. Point mutation of the conserved ATP binding site lysine abolished this complementation. This mutation in SWI2 exerted a dominant negative effect on transcription in yeast. A lysine to arginine substitution at the corresponding residue of BRG1 also generated a transcriptional dominant negative in human cells. BRG1 is exclusively nuclear and present in a high *M_r* complex of about 2 × 10⁶. These results show that the SWI2 family DNA-dependent ATPase domain has functional con-

servation between yeast and humans and suggest that a SWI/SNF protein complex is required for the activation of selective mammalian genes.

Activation of messenger RNA transcription in eukaryotes requires a complex array of protein–protein and protein–DNA interactions^{6,7}. SWI2 (also known as SNF2)^{8–12} is one of a group of activators distinct from both general RNA polymerase II factors and sequence-specific DNA binding proteins. SWI2 assists DNA-binding regulatory factors, including GAL4 (ref. 1), *Drosophila* bicoid, fushi-tarazu^{1,11} and mammalian glucocorticoid receptor (GR)², to activate transcription of their target genes in yeast. Suppression of *swi2* mutations by certain mutations in histone genes suggest SWI2 does this by overcoming the repressive effects of chromatin (C.L.P. and I. Herskowitz, unpublished results). Consistent with this view, mutation of SWI2 alters chromatin structure at yeast promoters *in vivo*¹³. SWI2 is a DNA-dependent ATPase³ and contains a set of six motifs characteristic of this function^{14–16}. SWI2 may function as a helicase^{14,15,17}; a model based on this potential activity¹⁸ suggests that binding of an activator to its DNA sequence targets the putative SWI2 helicase complex to promoter sites where it disrupts chromatin structure. Two findings indicate that functional homologues of SWI2 exist in higher eukaryotes. First, an activator of homeotic genes in *Drosophila*, *brahma* (*brm*) is strikingly related to SWI2^{4,5}. Second, the mammalian GR requires SWI proteins to activate transcription both *in vivo* in yeast and *in vitro* in *Drosophila* nuclear extracts². Although two human genes with some sequence similarity to SWI2 have been previously identified, ERCC6 (ref. 19) and hSNF2L (ref. 20) homology is limited solely to the region around the DNA-dependent ATPase motif.

These findings prompted us to search for functional human homologues of *brm* and SWI2. By screening a human HeLa cell cDNA library with a *brm* cDNA probe, we isolated a human gene, BRG1 (*brm*/SWI2-related gene 1) which encodes a 1,613 amino-acid protein (Fig. 1) highly related to *brm* (52% identity). Four domains of BRG1 and *brm* are particularly highly related (Fig. 2a, b). The first domain is extremely rich in proline (~25% proline in both proteins). The third and longest domain contains

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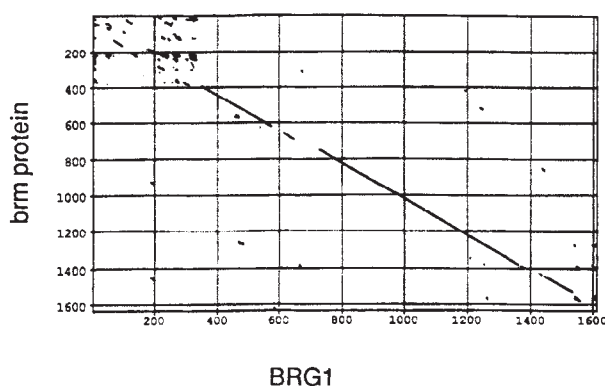
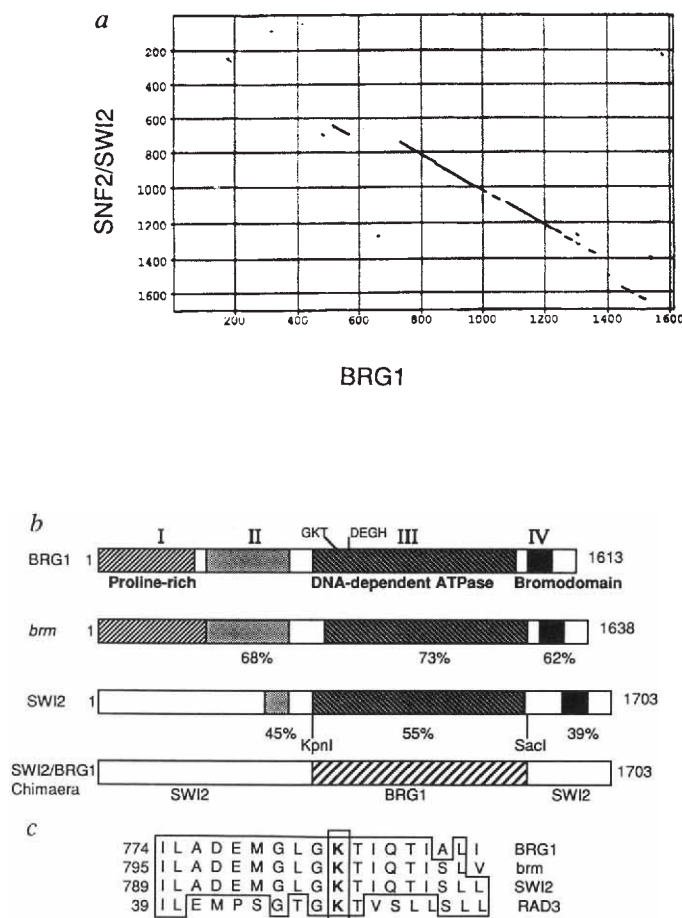


FIG. 2 Extent of amino-acid homology of BRG1 to SWI2 and brm. **a**, Protein matrix analysis comparison of SWI2 and brm to BRG1. Regions of amino-acid homology were quantified using a pam250 scoring matrix with a window size of 20 amino acids and a minimum homology score of 35% identical amino acids or conservative substitutions²⁶. The scatter seen in the N-terminal portion of the comparison between brm and BRG1 is due to the proline-rich composition of domain I (27.8% proline residues in the first 399 amino acids of BRM and 25.3% in the first 336 of BRG1). **b**, Conserved regions I-IV found in BRG1, BRM and SWI2. In addition to the proline-rich N-terminal sequences found in BRM and BRG1, three conserved regions are noted with per cent identity to BRG1 noted below each domain. BRG1 domains extend across the following amino acid residues: domain II, 350-571; domain III, 728-1388; domain IV, 1,447-1,523. The GKT sequence of the ATP binding motif and the DEGH sequence are noted. Also shown are the SWI2 KpnI and SacI sites denoting the boundaries of the SWI2 sequence replaced with BRG1 in the SWI2/BRG1 chimaera. **c**, Nucleotide binding motif comparison of the RAD3 helicase and SWI2 family proteins. Identical amino acids are boxed. The lysine required in RAD3 and SWI2 ATPase activity along with the corresponding residues in BRG1, brm and SWI2 is double boxed and shown in bold. This residue was mutated to an alanine in the Lys 798 mutants generated in the SWI2/BRG1 chimaera and in SWI2 and was mutated to an arginine in BRG1.

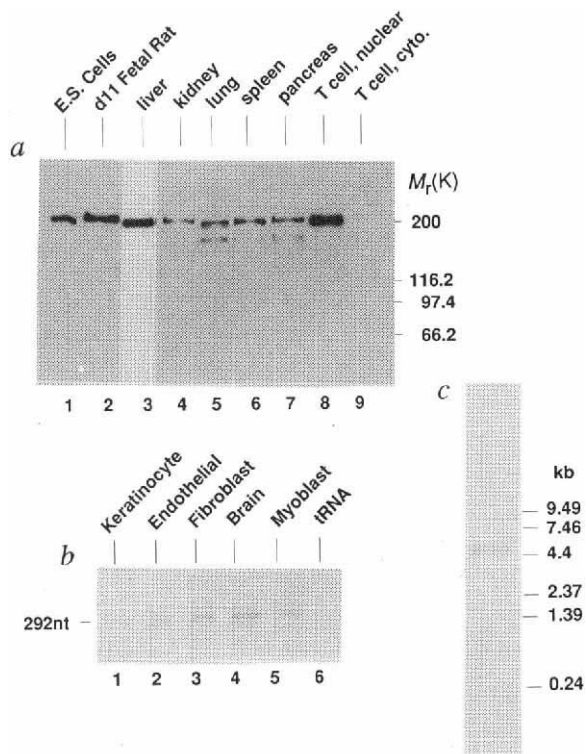


FIG. 3 Tissue expression of BRG1. **a**, Western analysis. Rabbit antisera J1 was raised to a 221 amino-acid C-terminal region of BRG1 fused to bacterial glutathione S-transferase and used in western analysis of cell extracts. Nuclear extract²⁷ (10 µg) was used from embryonic stem cells, fetal rat tissues or adult murine tissues as shown. Also loaded were 25 µg human T-cell fractionated nuclear and cytoplasmic extract²⁷ with Ponceau-S staining used (data not shown) to confirm identical amounts of transferred protein. Varying degrees of degradation of BRG1 has been consistently seen in extracts from pancreas, spleen and lung. **b**, Ribonuclease protection analysis of human brain tissue and primary keratinocyte, myoblast, fibroblast and endothelial cell cultures. RNA was prepared from human tissues and primary cultures and analysed using³²P-labelled antisense RNA transcribed using T7 polymerase over BRG1 nucleotide positions 3,336 to 3,628 (ref. 28). **c**, Northern analysis. Messenger RNA was isolated from human T cells and subjected to northern analysis²⁸ using a probe synthesized over nucleotide positions 3,336-3,984.

mutants, we examined the yeast *HO* gene as well as mammalian GR-driven transcription. In both cases the *SWI2/BRG1* chimaera restored capacity for transcriptional activation (Fig. 4b).

A point mutation was then introduced in both the chimaera and in wild-type yeast *SWI2* in the A box of the ATP binding motif at *SWI2* Lys 798 (Fig. 2c). This residue corresponds to the lysine required for ATP hydrolysis and helicase activity by the yeast *RAD3* helicase²⁵ and is necessary for ATPase activity and transcriptional activation by *SWI2*³. This mutant, along with the *SWI2/BRG1* chimaera also mutated at Lys 798, failed to restore normal growth to *swi2*⁻ cells (Fig. 4a), even though mutant protein expression was equivalent to wild type by western analysis (data not shown). These mutants also failed to restore transcriptional capacity to *swi2*⁻ cells (Fig. 4b). Furthermore, in *SWI2*⁺ wild-type cells, the overexpressed *SWI2* Lys 798 mutant exerted a dominant negative effect on GR-driven transcription (Fig. 4c).

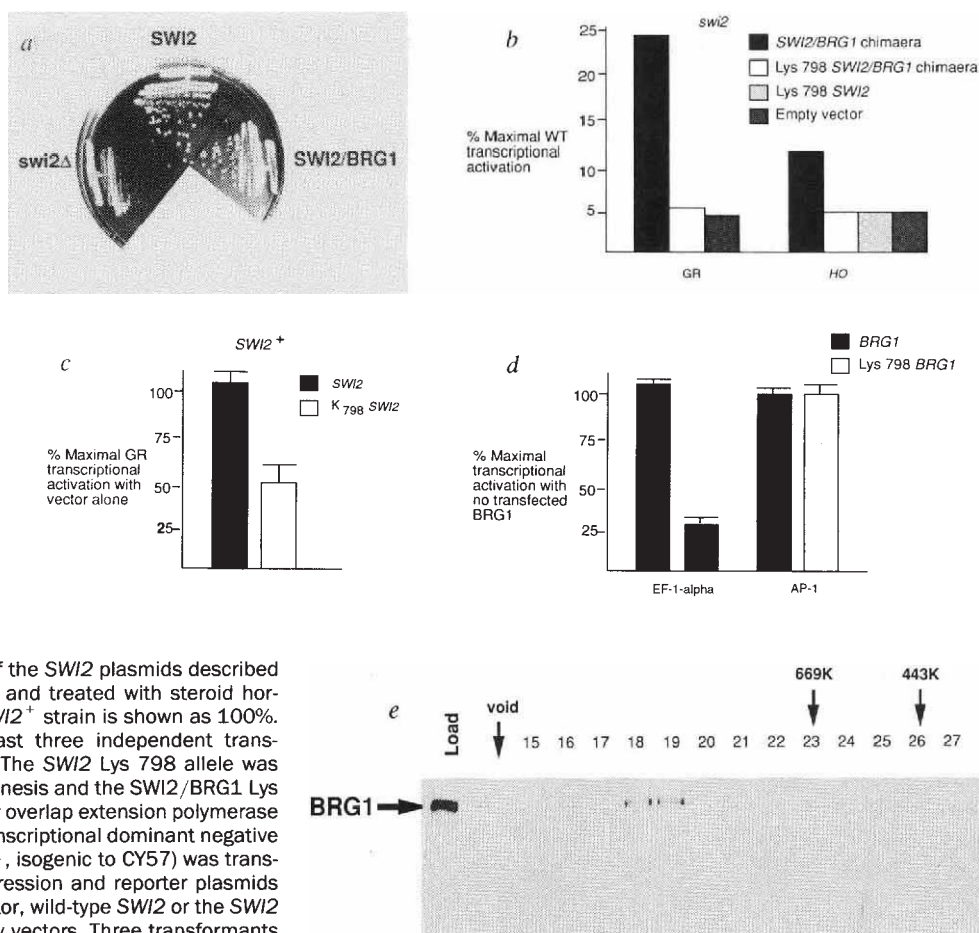
Transfection of a full-length mutant *BRG1* in which the lysine corresponding to *SWI2* Lys 798 was changed to an arginine reduced reporter gene expression directed by the EF-1 α promoter but not AP-1 or GAL4-VP-16 (Fig. 4d and data not shown).

FIG. 4 Effect of *BRG1* on growth and transcriptional activation in yeast and human cells. **a**, Growth complementation. Strain CY120, isogenic to CY26 (ref. 1), (α *swi2* Δ ::*HIS3 HO-lacZ*) was transformed with high-copy plasmids containing *SWI2*, *SWI2/BRG1*, or no insert. Transformants were streaked on minimal media and growth was assessed after 3 days at 30 °C. **b**, Complementation of *swi2*⁻ transcriptional defects. Strain CY120 was transformed with high-copy plasmids containing either a *SWI2*, *SWI2/BRG1* or *SWI2/BRG1* Lys 798 gene. The *SWI2* Lys798 allele was integrated at the *URA3* locus. Transformants were grown in liquid medium and assayed for expression of the *HO-lacZ* gene as previously described⁸. For measurement of GR activation, strain CY57 (α *swi2* deletion) was transformed with three plasmids: (1) a GRE-*lacZ* reporter plasmid pA26.x, that contains three GREs that drive expression of β -galactosidase⁴; (2) a plasmid that expresses full-length GR, pN795-*LYS2*, a derivative of pG-N795¹ (3) one of the *SWI2* plasmids described in **b**. Cells were grown in liquid cultures and treated with steroid hormone as described¹. Activation in the *SWI2*⁺ strain is shown as 100%. Data shown are the average of at least three independent transformants; variation was less than 10%. The *SWI2* Lys 798 allele was constructed by M13 site-directed mutagenesis and the *SWI2/BRG1* Lys 798 allele was constructed in triplicate by overlap extension polymerase chain reaction (PCR). **c**, *SWI2* Lys 798 transcriptional dominant negative effect in yeast cells. Strain CY26 (α *SWI2*⁺, isogenic to CY57) was transformed with three plasmids; the GR expression and reporter plasmids described above and either a control vector, wild-type *SWI2* or the *SWI2* K798 mutant contained on 2 μ high-copy vectors. Three transformants were treated and analysed as described above. **d**, *BRG1* Lys 798 transcriptional dominant negative effect in human cells. TAG Jurkat cells²⁹ (5×10^6) were transfected by electroporation with 2 μ g AP-1 luciferase³⁰ or EF-1 α luciferase³¹ reporter constructs and 2 μ g wild-type or mutant *BRG1* in the pBJ5 replicating overexpression vector as described²⁸. Mutant *BRG1* was constructed by overlap extension PCR and, along with the wild-type cDNA, was inserted as a 5' Sall to 3' EcoRI fragment into the *XhoI* and *EcoRI* sites of pBJ5. GAL4-VP16 (20 ng) in pBJ5 and 400 ng GAL₅ E1B-CAT were cotransfected as an internal control of transfection efficiency²⁸. *BRG1* wild-type and mutant proteins were epitope tagged at the C terminus and western analysis on extracts²⁹ from transfected cells confirmed similar levels of full-length expression of both proteins (data not shown). 16 hours after transfection,

This finding with mutant *BRG1* in human cells, and the similar effects of *SWI2* Lys 798 seen with GR in yeast, suggest that such a dominant negative effect may be due to formation of nonfunctional activator complexes at specific promoter sites leading to a failure of transcription initiation and suggest that *BRG1*, like *SWI2*^{8,9}, may influence the expression of a discrete subset of genes.

In yeast, *SWI2* is a component of a large multisubunit complex that contains at least four other *SWI/SNF* polypeptides (C.L.P., A. Dingwall and M. P. Scott, manuscript in preparation). To determine if *BRG1* also exists in a protein complex, human nuclear extract was fractionated on a fast protein liquid chromatography (FPLC) Superose 6 gel filtration column and elution of *BRG1* detected by western analysis (Fig. 4e). *BRG1* elutes in fractions 18–20, indicating an apparent *M_r* of 2×10^6 . This elution position is nearly identical to that observed for the yeast *SWI/SNF* protein complex (C.L.P., A. Dingwall and M. P. Scott, manuscript in preparation).

These findings identify a human gene highly homologous to *SWI2*, indicate that the function of the *SWI2* family DNA-dependent ATPase motif is evolutionarily conserved and depen-



dent on an intact ATP binding sequence and, furthermore, suggest that *BRG1* and *SWI2* may play similar roles in the activation of gene transcription. □

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Transposition of group II intron *aII* in yeast and invasion of mitochondrial genes at new locations

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INTRON mobility at the RNA level^{1–8} by splicing reversal at allelic (homing) and non-allelic locations (transposition) has been reported *in vitro*^{9–12}. In the living cell, however, only intron homing by unidirectional gene conversion has been described^{5,6,13,14}. Supposing that intron insertions at non-allelic sites might occur *in vivo*, we speculated that group II splice-site-associated macro-deletions^{15–18} in fungal mitochondrial DNA might result from group II intron transposition to new locations followed by recombination. We used polymerase chain reaction techniques to detect this critical, infrequent intermediate in mtDNA populations. Here we report on group II intron *aII* transposition to non-allelic, splicing-compatible locations within the *cox1* gene of yeast mtDNA. The identified integration sites are preceded by motifs similar to the upstream exon *A1*. Sequences flanking intron *aII* are not co-converted to the insertion sites and *cis*- and *trans*-acting mutations within *aII* reduce intron mobility below detection levels. These findings suggest the involvement of an RNA intermediate in group II intron transposition.

In the mitochondrial *cox1* gene from *Saccharomyces cerevisiae* (Fig. 1a), reverse transcriptase-like proteins¹⁹ encoded by group II intron²⁰ *aII* and/or *aI2* have been proposed to contribute to intron loss²¹, intron mobility and to site-specific mtDNA rearrangements^{3–6,22}. A certain class of site-specific deletions in

the *cox1* gene (del-B)¹⁸ has been reported to involve splice-site sequences of group II intron *aII*. Del-B macro-deletions extend from the authentic 3' splice-site of intron *aII* to downstream locations (B': *aI5a*₍₄₂₇₎; C': *aI5b*₍₄₈₃₎; and D': *aI5b*₍₈₄₈₎) which are preceded by sequence motifs similar to the 5' exon *A1* (IBS1- and IBS2-like)²³ of intron *aII* (Fig. 1a). We formulated the hypothesis of an intron *aII* transposition by splicing reversal at non-allelic, IBS-like locations as the initial stage in del-B formation. Further we speculated that after complementary DNA synthesis and recombination, homologous DNA recombination between direct repeats of the *aII* intron at the donor and at the recipient site could produce the observed macro-deletions.

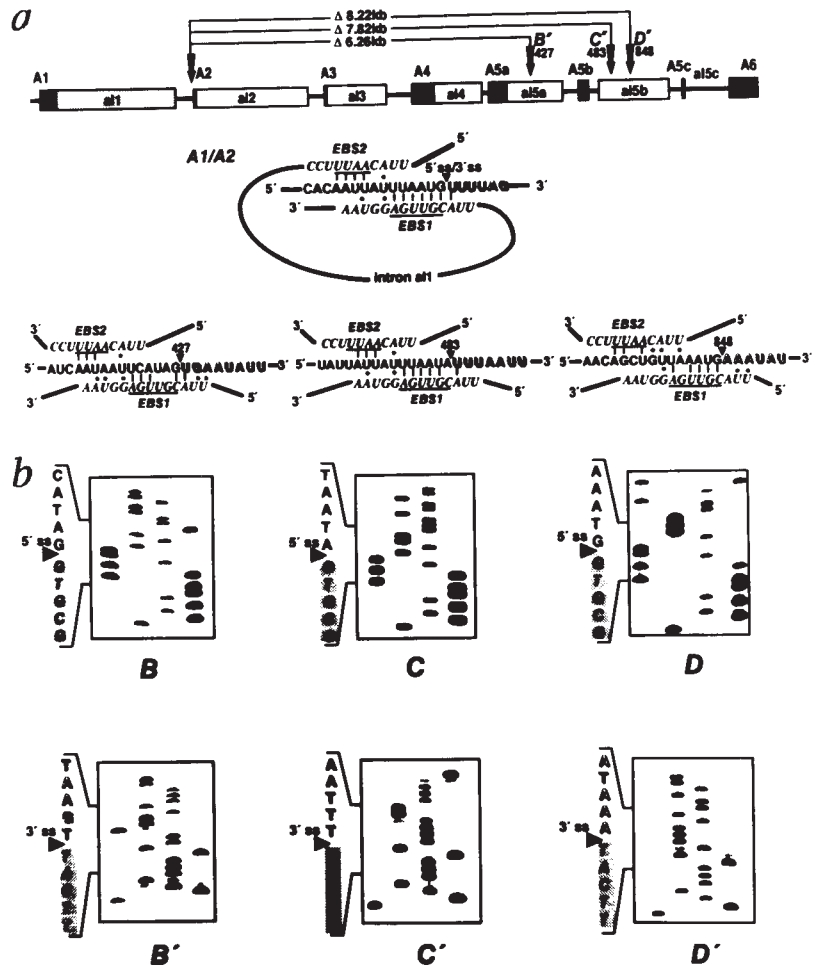
First we screened the *cox1* gene of wild-type mtDNA (*DBY-747*) by polymerase chain reaction (PCR) techniques, focusing on the occurrence of *aII* insertions at genetically observed del-B deletion end-points (B': *aI5a*₍₄₂₇₎; C': *aI5b*₍₄₈₃₎; and D': *aI5b*₍₈₄₈₎) (Fig. 1a). PCR amplification of mtDNA with primer sets selective for the respective 5' (B, C and D) and 3' junction fragments (B', C' and D') followed by dideoxy-sequencing revealed the site-specific intron insertion downstream of the IBS-like sequence motifs (Fig. 1b). On the basis of this we predicted that an IBS-like sequence is one major criterion for group II transposition. To confirm this we designed primers specific for *aII* insertions at three other IBS-like motifs in group II intron *aI5c*. Screening wild-type mtDNA by PCR followed by dideoxy-sequencing revealed the site-specific *aII* integration at all three IBS-like locations (*aI5c*₍₂₀₈₎, *aI5c*₍₂₇₂₎ and *aI5c*₍₃₂₀₎, respectively) (shown for *aI5c*₍₂₇₂₎ (E'); compare with Fig. 2).

We tested the co-existence of intron *aII* at both the donor and the recipient site. Focusing on the insertion at location *aI5c*₍₂₇₂₎ (E') (Fig. 2), we screened for the existence of a DNA-fragment flanked by two copies of *aII* sequences. For technical reasons we analysed mtDNA of strain 777–3A/del-B *aI5b*₍₈₄₈₎ in which the putative pair of introns would be separated by 1,059 base pairs (bp). The PCR product obtained, corresponding in size to the expected length of 1,458 bp (Fig. 2), was subjected to re-amplification with nested primers selective for unique junction fragments ((D'): 3' *aII/aI5b*; (E): 5' *aI5c/aII*; and (E'): 3' *aII/aI5c*, respectively). Results obtained by dideoxy-sequencing confirmed the existence of two copies of intron *aII* in the same DNA fragment (Fig. 2).

Sequence analysis of isolated junction fragments (D and D', respectively), which were obtained after a cloning step, revealed that *aII* transposition is conservative with respect to sequences flanking the integration site. This finding is important, because DNA-based group I intron homing involves co-conversion of sequences flanking the intron at frequencies that decrease with increasing distance from the intron border⁵. A further distinction between group I intron homing and group II intron transposition can be drawn. Sequences upstream of the group II insertion site can potentially base-pair at the RNA-level with the internal guide sequence (IGS: EBS1 and EBS2)²³ of intron *aII*, whereas sequences downstream of the insertion border showed almost no similarity to the 3' exon context of *A2* or as compared to another (compare Fig. 1a and 2). On the basis of this we predicted that group II intron transposition is RNA-mediated, probably through reverse splicing.

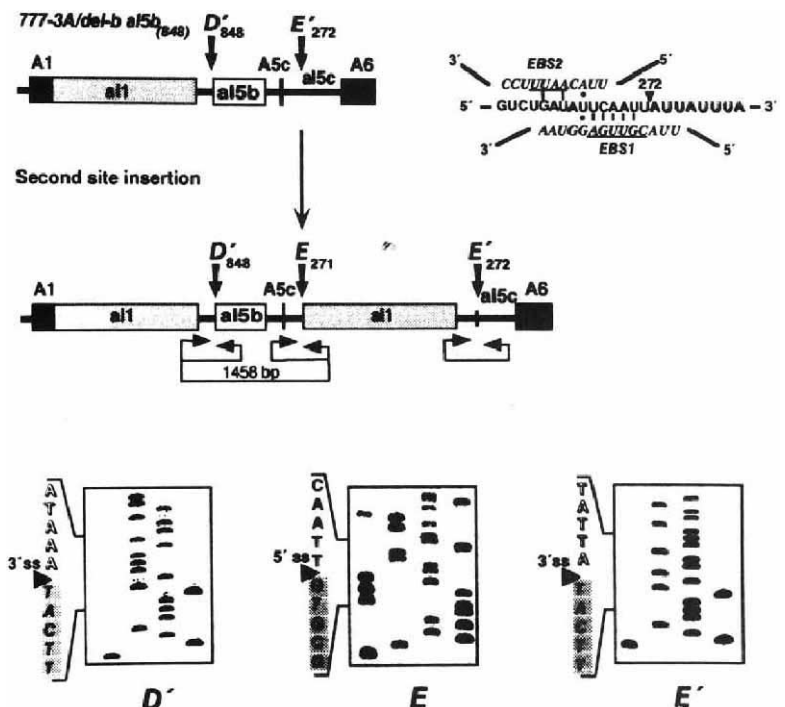
Evidence for this formulated hypothesis is twofold. First, group II mobility was found to be dependent on splicing-competent intron *aII* (Fig. 3). *Cis*- or *trans*-acting mutations (G2166 and V281, respectively)²⁴ within intron *aII* reduced intron transposition below detection levels in PCK studies. This suggests that group II intron transposition is not only prevented by a *trans*-acting mutation affecting the encoded RNA maturase (V281) but also by a *cis*-acting one (G2166) which leaves the expression of a functional RNA maturase intact but interferes with the productive folding of intron *aII* RNA sequences. Essentially the same observations have been made by Meunier *et al.*¹³, analysing group II intron *aII* and *aI2* homing in genetic crosses. It should be stressed that these data provide a further distinction

FIG. 1 a, Physical map of the mitochondrial *cox1* gene (long-form) of *S. cerevisiae* and characterized *al1* insertion sites (B', C', D'). The deletion end-points of *del-B* mit⁻ mutants are marked by arrows. Exon sequences are indicated by filled boxes and reading frames of introns (ORFs) are shown as open or shadowed boxes. Insertion sites are numbered according to the position number of the nucleotide adjacent to the IBS-like sequences in the respective introns. Potential base-pairing interactions (EBS1/IBS1 and EBS2/IBS2)²³ (EBS: underlined) of intron *al1* (italics) at the RNA level with its canonical ligated exons (A1/A2) and with IBS-like sequences upstream of the insertion sites (B': 427; C': 483; and D': 848) are indicated; arrowheads mark the integration junctions. b, Dideoxy-sequencing of 5' (B, C and D) and 3' junction fragments (B', C' and D') obtained after PCR amplification. Intron *al1* sequences (italics) are shown in shadowed boxes; the insertion borders, similar to 5' and 3' splice-site of *al1*, are marked by arrowheads; sequencing lanes G, A, T and C: left to right. METHODS. Yeast wild-type strain DBY-747 was grown in YPD medium supplemented with 2% glucose. Cells were collected at late logarithmic phase and mtDNA was prepared as previously described²⁹. 5' Junction fragments were obtained by PCR amplification of total mtDNA with an oligonucleotide (1) complementary to the 5' part of intron *al1* in conjunction with 5' terminally biotinylated plus strand primers (2, 3 and 4, respectively), located upstream of the proposed insertion sites (B': *al5a*₍₄₂₇₎ (2); C': *al5b*₍₄₈₃₎ (3); and D': *al5b*₍₈₄₈₎ (4)). 3' Junction fragments were obtained by PCR amplification with an *al1* internal plus strand primer (5), located in the 3' region of intron *al1*, in conjunction with 5' terminal biotinylated minus strand primers (6, 7 and 8, respectively) located downstream of the insertion sites (B': *al5a*₍₄₂₇₎ (6); C': *al5b*₍₄₈₃₎ (7); and D': *al5b*₍₈₄₈₎ (8)). Direct solid-phase sequencing of amplified reaction products using magnetic beads as solid support was performed as described³⁰. PCR primers: 1, position 86–106 in *al1*–; 2, position 312–336 in *al5a*–; 3, position 93–112 in *al5b*–; 4, position 688–709 in *al5b*–; 5, position 2,178–2,203 in *al1*–; 6, position 657–680 in *al5a*–; 7, position 688–711 in *al5b*–; 8, position 1,005–1,030 in *al5b*–. Sequencing



primer for 5' junction fragments (B, C and D) (1); for 3' junction fragments B', C' and D', position 2,363–2,380 in *al1*–.

FIG. 2 Physical map of the *cox1* gene of respiratory deficient mit⁻ mutant 777-3A/*del-B al5b*₍₈₄₈₎¹⁸ and characterized second-site insertion of *al1* within group II intron *al5c*₍₂₇₂₎. Exon sequences are indicated by filled boxes, ORFs of introns are shown as open or shadowed boxes. Arrows mark the *al1* donor (D') and the insertion site (E'). The potential base-pairing interactions at the RNA-level of intron *al1* (italics) with *al5c* sequences at the insertion site (E') are indicated. Junction fragments (D', E and E') obtained by PCR amplification were characterized by dideoxy-sequencing³⁰ essentially as described in Fig. 1. Intron *al1* sequences (italics) are shown in shadowed boxes; 5' and 3' splice-site borders of *al1* are marked by arrowheads; sequencing lanes G, A, T and C: left to right. METHODS. Strain 777-3A/*del-B al5b*₍₈₄₈₎ was grown in YPD medium supplemented with 2% glucose. Cells were collected at late logarithmic phase, and mtDNA was prepared as described²⁹. Primers for PCR amplification: D' (5), position 2,178–2,203 in *al1*–; and 8, position 1,005–1,030 in *al5b*–. E (1), position 86–106 in *al1*–; and 9, position 9 in *al5c*– to position 3 in *al5c*–. E' (5), position 2,178–2,203 in *al1*–; and 10, position 313–335 in *al5c*–. Dideoxy-sequencing primer for the 5' junction fragment E (1); for 3' junction fragments (D' and E') position 2,363–2,380 in *al1*–.



between group I homing and group II intron transposition, because a *cis*-acting mutation which leaves the expression of the group I intron-encoded DNA-endonuclease intact has been reported not to abolish gene conversion^{25,26}. Second, *all* insertion at the RNA level by reverse splicing predicts compatibility with subsequent splicing at new locations³. This prerequisite for an RNA-based intron movement was confirmed by northern analysis of mit⁻ 777-3A/*del-B* pre-mRNAs (*al5a*₍₄₂₇₎, *al5b*₍₄₈₃₎ and *al5b*₍₈₄₈₎) in which the excised *all* lariat intron was detected. Dideoxy-sequencing of cDNA products across the ligated exon junctions (*A1:al5a*₍₄₂₇₎, *A1:al5b*₍₄₈₃₎ and *A1:al5b*₍₈₄₈₎) revealed the site-specific exon ligation in the respective 777-3A/*del-B* mutant strains. In addition, the occurrence of these RNA products was also observed in wild-type yeast 777-3A mtRNA preparations (data not shown).

Composite introns in which a group II intron is inserted into a group I or another group II intron as observed in this study bear striking resemblance to reports on evolutionary fixed 'twintrons' in *Euglena* chloroplast DNA (ctDNA)²⁷. In both cases, the inserted introns have been shown to be splicing-competent at their new locations.

Our results provide evidence for the transposition of group II intron *all* to non-allelic sites in mtDNA. Although it is premature to draw definitive conclusions, our data are strongly suggestive to group II intron mobility at the RNA level by splicing reversal. Reverse transcription followed by recombination of the chimaeric cDNA product may fix the transposed intron at the DNA level. It is worth mentioning that intron *all* and/or *al2* actually have been reported recently to encode a reverse transcriptase¹⁹ in yeast mitochondria. The possibility remains that the *all* lariat intron could integrate directly into short tracks

of RNA or single-stranded DNA at the replication fork⁷ and an RNA-mediated conversion of the recombinant RNA product might result²⁸. The discrimination between these and other possibilities merits further investigations. □

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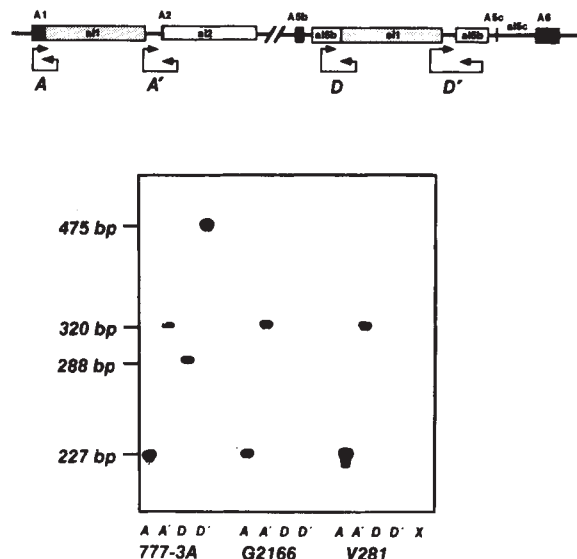


FIG. 3 *Cis*- (G2166) and *trans*-acting mutations (V281) within intron *al1* reduced intron transposition below detection levels in PCR studies. Junction fragments D (288 bp) and D' (475 bp) generated by PCR amplification in the case of transposition of intron *al1* into intron *al5b* at position 848 are indicated. As a control, the 5' (A, 227 bp) and 3' (A', 320 bp) intron *al1* border fragments of wild-type 777-3A and mutant strains (G2166 and V281)²⁴ were selectively amplified in parallel. X, Negative control.

METHODS. Total preparations of mtDNA (500 ng) were subjected to PCR amplification in the presence of [γ -³²P]dATP (2 μ Ci; 3,000 Ci mmol⁻¹) with primer sets (20 pmol each) specific for the respective junction fragments (A, A', D and D'). Reaction products were fractionated on 7.5% polyacrylamide, 8M-urea gels and autoradiographed. Primer sets: A: 11, position 74-92 in *A1*+ and 1, position 86-106 in *A1*-; A': 5, position 2,178-2,203 in *A1*+; and 12, position 6-29 in *A2*-; D: 4, position 688-709 in *A5b*+; and 1, position 86-106 in *A1*-; D': 5, position 2,178-2,203 in *A1*+; and 8, position 1,005-1,030 in *A5b*-.

Transposition of a group II intron

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AMONG mobile genetic elements, self-splicing introns are of particular interest. They belong to either group I or group II depending on their three-dimensional structure¹⁻³. Homing, the systematic intron invasion of an intronless gene when it encounters its homologous intron-bearing allele, is the only means for intron mobility so far demonstrated⁴. It depends on the activity of the intron-encoded protein and is very specific for the acceptor site⁴⁻⁷. Intron transposition, the transfer of an intron to a novel site, predicted on the basis of phylogenetic studies^{8,9} and *in vitro* reverse-splicing experiments, has been proposed to be responsible for evolutionary intron spreading^{2,10-14}. Here we present results from polymerase chain reaction experiments consistent with transposition of a group II intron. This event is proposed to account for the site-specific deletion in the mitochondrial chromosome of the fungus *Podospira anserina* that is associated with the premature death syndrome¹⁵ and might also be involved in the senescence process¹⁶ affecting this species.

Instability of the mitochondrial chromosome in the fungus *Podospira anserina* is responsible for two degenerative syndromes. Senescence is accompanied by the extrachromosomal amplification of intron alpha (α), the first intron of the mitochondrial *cox1* gene, as Sen DNA α (refs 17-20). Premature

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death syndrome, under the control of two nuclear genes, is characterized by a site-specific deletion; the two breaks take place at the 5' end of intron α on one side and between the genes encoding the transfer RNA for isoleucine (tRNA^{Ile}) and the tRNA for serine (tRNA^{Ser}) genes on the other side (Fig. 1a, b; positions 825–38,670)¹⁵. The mechanisms responsible for these rearrangements are still unknown. But the involvement of intron α in both degenerative processes led us to suspect the mobility of this self-splicing group II intron^{17,21}. We reasoned that if intron α were able to integrate between the tRNA^{Ile} and tRNA^{Ser} genes, homologous recombination between the two copies of intron α

would lead to the deleted mitochondrial chromosome which is abundant in the prematurely dying mycelia (Fig. 1b).

We looked for molecules containing a mislocated intron α by polymerase chain reaction (PCR) experiments using as primers several pairs of oligonucleotides (Fig. 1c). We first examined mitochondrial DNA extracted from prematurely dying mycelia. Attempts to amplify a DNA fragment containing the entire intron α (2,539 base pairs (bp) long) between the two tRNA genes were unsuccessful. This was not surprising, because a more abundant template, the wild-type chromosome, allowed the amplification of a shorter fragment (Fig. 1a, c; primers 1 and

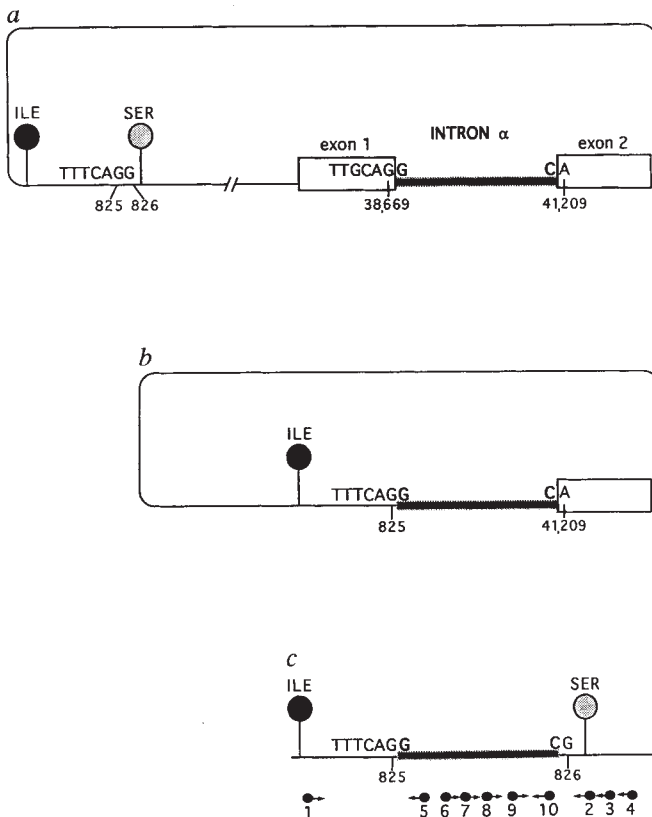


FIG. 1 a, Mitochondrial chromosome of the young wild-type strain, a circular 94-kb DNA molecule completely sequenced (EMBL: Mtpacg; accession number X55026)³⁰. The natural senescence process affecting all wild-type strain cultures and resulting in apical cell death, is concomitant with the amplification of intron α (bold characters and lines) as extrachromosomal multimeric circular DNA molecules (Sen DNA α). The long-lived *mex16* mutant, selected as escaping from a senescent culture of the currently used *s* wild-type strain, contains a mitochondrial chromosome rearranged in the region overlapping the exon 1/intron α junction (38,640–38,692)²². Boxed regions refer to *cox1* exons, $\uparrow$ to tRNA genes. TTGCAG (G, position 38,669) and TTTCAG (G, position 825) stand for IBS1 and IBS1-like DNA sequences, respectively. b, Deleted mitochondrial chromosome, a 57-kb DNA molecule found, together with the wild-type chromosome, in heteroplasmic mycelia of the AS1-4 *Rmp*⁻ mutant that undergo premature death. AS1-4 is a mutant allele of the nuclear AS1 gene encoding a ribosomal cytosolic protein, *Rmp*⁻ is an unidentified gene tightly linked to the mating-type minus locus¹⁵. AS1-4 *Rmp*⁺ is a long-lived mutant, the mitochondrial chromosome of which reveals no difference to the wild-type one. c, Transposed intron α deduced from PCR experiments using different pairs of primers: primers 6 + 4, 7 + 4, 8 + 3, 9 + 4, 9 + 3, 9 + 2 were used to look for the intron α /tRNA^{Ser} junction, primers 1 + 4 to look for the entire intron α at its ectopic location and primers 1 + 5 to detect, in the wild-type strain, the tRNA-Ile/intron α junction already characterized in the AS1-4 *Rmp*⁻ mutant strain¹⁵. Synthetic oligonucleotides used as primers or probes (●) are numbered according to their position on the wild-type genome³⁰. The arrows indicate their 5'–3' orientation. Position 675 is the 5' nucleotide of primer 1, 860 for primer 2, 868 for primer 3, 935 for primer 4, 38,774 for primer 5, 39,140 for primer 6, 40,038 for primer 7, 40,568 for primer 8, 40,887 for primer 9 and 41,120 for primer 10.

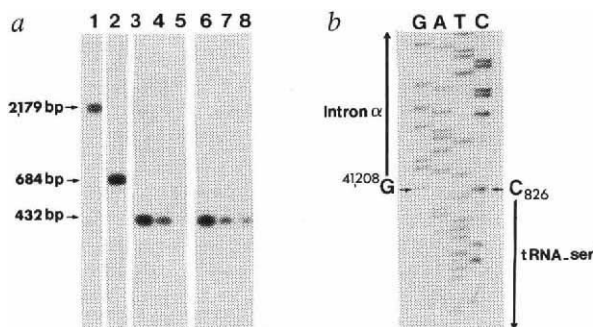


FIG. 2 a, RNase-treated mitochondrial DNA samples from AS1-4 *Rmp*⁻ and *s* wild-type strains were diluted 1:5, 1:10, 1:100 (lanes 6, 7, 8, respectively) and 1:2, 1:5, 1:50 (lanes 3, 4, 5, respectively) to do PCR experiments using primers 9 + 4 (exact position of the different primer is given in Fig. 1). The *s* wild-type strains of either mating type and AS1-4 *Rmp*⁺ mutant strain, that do not express the premature death syndrome, gave identical results. Amplification from primers 6 + 4 (lane 1) and 8 + 3 (lane 2) were obtained from AS1-4 *Rmp*⁻ mitochondrial DNA samples diluted 1:10. Amplified DNA fragments were loaded on 1.2% agarose gels and Southern blot analysis was done with 5'-³²P-labelled primer 10 as the probe. b, The 432-bp amplified DNA fragment was purified from a sea-plaque-agarose gel and cloned into a PTZ 18 phagemid. Several independent clones were analysed. Sequence of the intron α /tRNA^{Ser} junction was initiated from the antisense primer 3 and done using the BRL Sequenase kit. The sequence overlapping the intron α /tRNA^{Ser} junction was determined on both strands. From the two ectopic cloned junctions, a total of 420 nucleotides of the 'transposed' intron was determined without detecting any single-base difference with the 'resident' intron.

METHODS. PCR experiments in a Hybaid programmable thermal reactor, were done as follows: 3' initial denaturation at 94 °C, 30 cycles: 1 min at 94 °C (denaturation), 5 s at 65 °C, 1 min at 5 °C below the lower fusion temperature of the oligonucleotides (annealing), 5 s at 65 °C, 1 min at 72 °C (polymerization) and finally one cycle of 10 min at 72 °C (final elongation).

2). The DNA fragment containing the junction of the tRNA^{lle} region with the 5' end of intron α , which is expected to result from the amplification of the abundant deleted chromosome, was indeed detected (Fig. 1b, c; primers 1 and 5). The most pertinent junction to identify, in order to demonstrate the presence of intron α at its ectopic location, was the intron α /tRNA^{Ser} junction. The lengths of the six DNA fragments, amplified from six different pairs of primers and their analysis by hybridization and sequencing (Fig. 2a, b) agreed with the expected ectopic location of intron α . Whatever the molecule carrying the intron α /tRNA^{Ser} junction, a short DNA molecule, a chromosome with two copies of intron α or a derived molecule (generated by homologous recombination at the same time as the deleted chromosome), it necessarily results from a previous ectopic integration of intron α .

Similar experiments were done on mitochondrial DNA from wild-type strains in which the deleted chromosome is undetectable by hybridization. Surprisingly, both pairs of primers (tRNA^{lle}/intron α and intron α /tRNA^{Ser}) allowed amplification of DNA fragments (Fig. 2a) whose sequences also agreed with the presence of intron α at the same ectopic location. Detection of the transposed intron in wild-type strains raises the question of whether the nuclear genes¹⁵ controlling premature death activate one of the steps leading to the deleted molecule, or positively select this molecule. In contrast, in mitochondrial DNA of the long-lived *mex 16* mutant²², rearranged in the 5' region of intron α (see legend to Fig. 1a), we failed to detect the expected ectopic integration. This supports the conclusion that the mislocated intron, present in all other strains, indeed resulted from a rare but recurrent transposition process rather than from its inheritance at the heteroplasmic state. Furthermore, heteroplasmy is not vegetatively stable and is systematically lost through the sexual process in *Podospora anserina*²³.

The ectopic insertion of intron α takes place downstream from a sequence bearing homology with the 3' end of its actual upstream exon (Fig. 1a), namely the intron binding site (IBS) sequence. At the RNA level, pairing of intronic EBS1 and exonic IBS1 sequences plays an important role in group II intron self-splicing²⁴. Furthermore, the presence of IBS1 was also shown to be sufficient to direct downstream integration of the intron in *in vitro* reverse splicing experiments using as a substrate either the ligated exons or unrelated RNA sequences^{14,25}. The mechanism leading to the ectopic insertion of intron α is thus consistent with a reverse splicing pathway, which is probably also used for group II intron homing⁷. In the case of *Podospora*, the high abundance²⁶ of the spliced intron α , and the high transcription rate of the tRNA region (containing the IBS1-like target sequence) could enhance the efficiency of the erroneous reverse splicing. Moreover, the tRNA^{Ser}, located just downstream from the RNA transposed intron, could be used as primer by the reverse transcriptase, leading to the DNA transposed intron we have detected. The erroneous reverse splicing mechanism would also account for the mitochondrial DNA rearrangements observed in senescent mycelia. The integration of intron α downstream from the actual IBS1 sequence would result in RNA tandem repetitions of the intron. Reverse transcription, which increases during senescence²⁷, followed by recombination, would lead to the abundant Sen DNA α circular molecules.

We have detected the presence of a group II intron at a novel location in the mitochondrial chromosome of *Podospora anserina*. Our results are consistent with a recurrent low-rate transposition of this intron which we were able to detect because it participates in a degenerative process. This finding constitutes strong support for intron transposition occurring *in vivo* and for the hypothesis that group II introns, supposed to be the ancestors of the nuclear splicing apparatus (snRNAs)^{28,29}, have spread during the course of evolution^{2,8,14,25}. □

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Tandem binding in crystals of a *trp* repressor/operator half-site complex

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THE crystal structure of *trp* repressor tandemly bound in a 2:1 complex to a 16-base-pair palindromic DNA containing a central *trp* operator half-site has been determined and refined to 2.4 Å resolution. Despite dramatically different DNA sequence contexts and crystallization conditions, the protein/DNA interface is essentially identical to that seen in the original *trp* repressor/operator complex structure¹. Water-mediated sequence recognition by *trp* repressor is likely to be related to the unusual end-on approach of the recognition helix (E), which allows sharing of the major groove by tandem dimers. The tandem complex model accounts for the mutational sensitivity of all *trp* operator base pairs. The structure also provides the first detailed view of the tandem interaction, revealing a key role for the amino-terminal arms.

The way in which DNA is bound by the *Escherichia coli trp* repressor, a key ligand-activated component in the transcriptional regulation of L-tryptophan synthesis, has been extensively studied by structural, genetic and biochemical methods (reviewed in refs 2–4). In the original 2.4-Å repressor/operator complex crystal structure¹ (here referred to by its PDB code, 1TRO), *trp* repressor, a dimer of two identical 12.5K subunits with two L-tryptophan ligands, binds with two-fold symmetry to a palindromic consensus operator (Fig. 1a). Although consistent with chemical protection results using natural *trp* operators⁵, unexpected features provoked questions about whether the structure was a specific recognition complex^{6,7,10}. High-resolution refinement⁴ confirmed, however, that genetically sensitive^{8,9}

base pairs 5, 6 and 7 are recognized through intervening water molecules. The extensive contact to the backbone of 1TRO DNA suggested¹ that indirect readout could account for genetic sensitivity^{8,9} of base pairs 2 and 4.

On the basis of the internal approximate dyad symmetry of the natural *trp* operator half-sites, it was proposed that an alternative duplex DNA containing a single central half-site was the minimal operator target of a single *trp* repressor dimer⁶. Double-label gel shift experiments^{10,11} showed, however, that whereas the consensus operator sequence binds a single repressor dimer, the alternative sequence binds two dimers with high cooperativity. A model in which two repressor dimers bind simultaneously with two-fold symmetry to the central half-site was therefore proposed^{10,11} (Fig. 1*b*).

We determined the crystal structure of *trp* repressor bound to the alternative sequence and have completed model refinement to 2.4 Å resolution (Table 1). The extensive interface between repressor and DNA in 1TRO is preserved in the tandem complex, including hydrogen-bonded contact to the genetically sensitive bases 5, 6 and -7 through intervening water molecules (Table 1 legend). The crystal structure features a superhelix of *trp* repressor dimers winding with left-handed screw symmetry around stacked 16-base-pair (bp) DNA duplexes (Fig. 1*c, d*); adjacent repressor dyad axes are 8 bp apart and rotated by 270° about the DNA helix axis. Superhelix formation is facilitated by formation of end half-sites (created by abutment of DNA duplexes) that are fortuitously in register with central half-sites

(Fig. 1*c*). Each repressor dimer links two DNA duplexes by binding to the central half-site of one duplex with one 'reading head'¹² (the D and E helices of one repressor subunit, labelled subunit c), and to an end half-site of an adjacent duplex with the other reading head (subunit e). Our crystal structure thus mimics the proposed¹³ tandem binding of repressor to the imperfect palindromic repeats found at 8-bp intervals in natural *trp* operator sequences.

In modelling studies¹ based on the 1TRO structure, a copy of the repressor dimer rotated by 180° about one of the putative tandem two-fold axes (open dyads in Fig. 1*a*) could be accommodated simultaneously with the first dimer, while preserving protein-phosphate contacts and avoiding stereochemical clashes. Only one direct tandem contact was possible: an ion pair between the side chains of Lys 90 and Asp 46 (subunits c+ and e, respectively, in Fig. 1*c*). Our refined structure reveals two important deviations from this simple model: a direct tandem contact mediated by the repressor amino-terminal arm, and, correlated with this, an alternative conformation of Asp 46e in which the side chain is rotated away from Lys 90c+ (Fig. 2).

In crystal structures of repressor^{14,15}, aporepressor¹² and 1TRO^{1,3,4}, the N-terminal arms (residues 2-15; ref. 14) are poorly ordered and in a variety of extended conformations making contacts mediated by crystal-packing arrangements. In the tandem complex, the arms instead adopt a 'bent elbow' conformation, with Tyr 7 at the elbow tip and residues 2-6 tucked into the space between the repressor and the minor groove of the

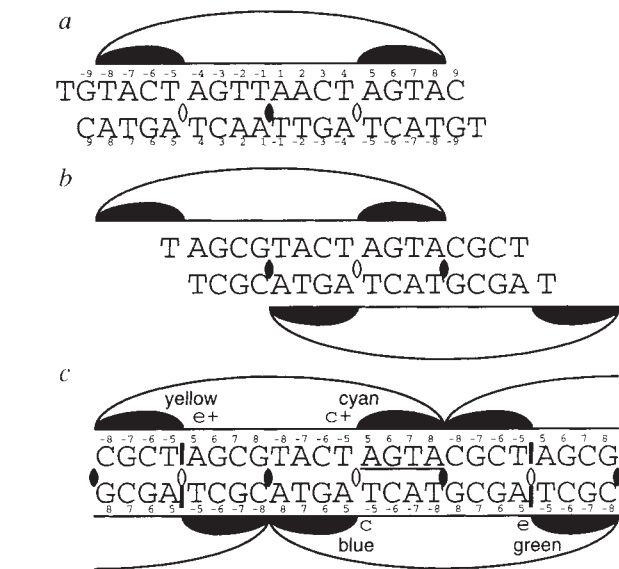


FIG. 1 DNA sequences and repressor binding motifs. *a*, The sequence of the 19-mer DNA duplex of 1TRO¹ is shown with a schematic diagram (similar to Fig. 1 of ref. 11) of the repressor dimer representing the symmetry and approximate span of the interaction (top line of the sequence is 5' to 3'). The two black slivers represent the two symmetrical reading heads of the repressor dimer. The original base numbering scheme¹ is shown. The filled and open dyad symbols indicate positions of repressor dimer and tandem two-fold axes respectively (α and β in ref. 6). *b*, The sequence of the 17-mer DNA duplex used in the present work is shown in an analogous schematic diagram of its proposed two-fold symmetric tandem interaction with two repressor dimers about the single central half-site^{10,11}. *c*, Schematic diagram showing end-to-end stacking of 2:1 tandem complexes. Thick vertical lines mark DNA duplex ends which abut to form additional two-fold symmetric half-sites (5' unpaired Ts are excluded from the stacking interaction). The unique repeating unit of the tandem complex is thus exactly one-half of a half-site, or a 'quarter-site', delimited by a tandem dyad and an adjacent repressor dyad (the two distinct quarter-site sequences, central (5'-AGTA-3'), and end (5'-AGCG-3'), are degenerate in our crystal structure because the DNA adopts two alternate positions which superimpose the two sequences). For our discussion we chose a reference quarter-site, underlined in the top strand. DNA base pairs are numbered 5-8 for ease of comparison with 1TRO^{1,3,4} (*a*; note that positions 2, 3 and 4 of 1TRO are equivalent to positions -7, -6 and -5). Subunits of two adjacent repressor dimers that form the main contacts with the reference quarter-site are labelled as c, e and c+, e+. Subunit colour labels are keyed to Fig. 2 (but not to Fig. 1*d*). *d*, CPK model of the tandem complex showing a left-handed superhelix of *trp* repressor dimers wrapped around stacked DNA duplexes. The view is of the crystal *ac* plane. Blue and green dimers straddle crystallographic two-fold axes. Between the two blue dimers (one crystallographic repeat along the *ac* short diagonal) there is one complete turn of the superhelix about two stacked 16-bp DNA duplexes, shown in magenta (phosphorus atoms) and grey (other atoms). The two duplexes together form exactly 3 helical turns of B-form DNA with 10.67 bp per turn. The rise per bp along the diagonal (103.5 Å/32 bp = 3.23 Å) is slightly shorter than ideal for B-form DNA (3.38 Å; ref. 28); the discrepancy is explained by bending of the helix axis at each half-site central TA step towards the major groove by ~15°, as in each half-site of the DNA duplex of 1TRO¹.

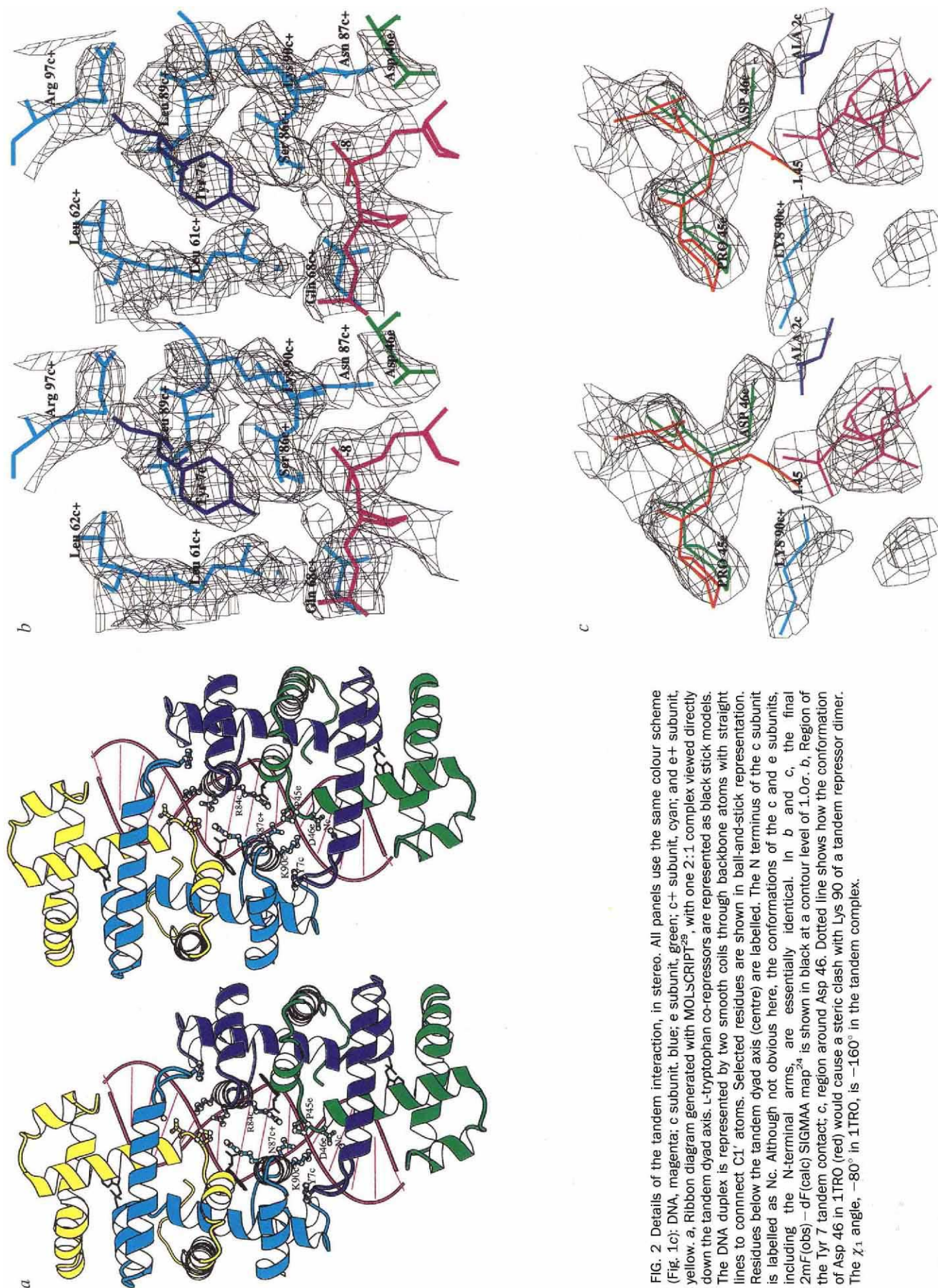


FIG. 2 Details of the tandem interaction, in stereo. All panels use the same colour scheme (Fig. 1c): DNA, magenta; c subunit, blue; e subunit, green; c+ subunit, cyan; and e+ subunit, yellow. a, Ribbon diagram generated with MOLSCRIPT²³, with one 2:1 complex viewed directly down the tandem dyad axis. b, L-tryptophan co-repressors are represented as black stick models. The DNA duplex is represented by two smooth coils through backbone atoms with straight lines to connect C1' atoms. Selected residues are shown in ball-and-stick representation. Residues below the tandem dyad axis (centre) are labelled. The N terminus of the c subunit is labelled as Nc. Although not obvious here, the conformations of the c and e subunits, including the N-terminal arms, are essentially identical. In b and c, the final 2mF(obs) - dF(calc) SIGMAA map²⁴ is shown in black at a contour level of 1.0σ. b, Region of the Tyr 7 tandem contact; c, region around Asp 46. Dotted line shows how the conformation of Asp 46 in 1TRO (red) would cause a steric clash with Lys 90 of a tandem repressor dimer. The χ₁ angle, -80° in 1TRO, is -160° in the tandem complex.

DNA near the repressor dyad. Tyrosine at position 7 mediates the most extensive direct contact between tandem repressor dimers (Fig. 2b). One face of the Tyr 7c aromatic ring is snugly seated in a hydrophobic basin formed on the surface of the tandem dimer by Leu 61, Leu 62, Leu 89, Lys 90 and Arg 97 of subunit c+. Tyrosine 7c also forms hydrogen bonds with Ser 86c+ O γ through its side-chain hydroxyl and Arg 97c+ N ϵ through its main-chain carbonyl oxygen. There is a second, less extensive hydrophobic interaction with the other side of Lys 90c+ by Pro 45c (Fig. 2a, b). All other tandem interactions in

TABLE 1 Crystallographic data

	5'-TAGCGTACT AGTACGCT-3'	5'-TAGCG' UACT AGTACGCT-3'	5'-TAGCGTAC' U AGTACGCT-3'
Resolution (Å)	2.4	3.0	3.0
Measured reflections	30,065*	24,401*	15,615†
Unique reflections	18,723	9,763	5,238
Completeness (1)	90.3	92.5	49.6
$R_{\text{sym}}^{\ddagger}$	0.050	0.083	0.095
R versus native§	—	0.121	0.106

Crystals of the tandem complex (space group C2, unit cell dimensions $a = 112.34$ Å, $b = 90.16$ Å, $c = 58.65$ Å, $\beta = 113.92^\circ$) were obtained as described¹⁸. Data were collected at the Brookhaven NSLS and processed with MADNES¹⁹. The structure was solved using the molecular replacement package MERLOT²⁰ with 1TRO¹, stripped of water molecules, as the search model. An equivalent molecular replacement solution was obtained with repressor dimer alone as search model. The crystallographic R factor ($(\sum |F_{\text{obs}} - F_{\text{calc}}|) / \sum F_{\text{obs}}$) after rigid body refinement was 44% for the 1TRO model (49% for repressor dimer). A new model was generated by replacing 1TRO DNA with B-form DNA of the appropriate sequence according to the alignment of Fig. 1a and c. Iodo-uridine derivatives verified that the base-pair registration of stacked 16-bp duplexes with respect to the repressor dyad axes was assigned correctly. Helical axes of the stacked DNA duplexes span the short diagonal of the crystal ac plane, so every other repressor dimer of the local 4 $_2$ 22 symmetry superhelix falls on a crystallographic two-fold axis. However, the DNA sequence about these repressor dyads violates crystal symmetry (Fig. 1c). The C2 asymmetric unit (a.u.), with one full repressor dimer, two repressor subunits and one DNA duplex, was therefore expanded to the P2 $_1$ a.u., with two full 2:1 complexes. Application of C2 symmetry operators to this doubled a.u. yields two overlapping structures with staggered DNA positions that satisfy observed crystal symmetry. The two 2:1 complexes of our model were subdivided into four equivalent units related by non-crystallographic symmetry (n.c.s.) operators, each containing one repressor dimer plus one strand of DNA duplex. Refinement with XPLOR²¹ first employed strict and then strongly restrained n.c.s., with individual repressor subunits restrained with additional n.c.s. terms. Relaxation of n.c.s. restraints was monitored by free R -factor calculations²². Manual adjustments of the model with TURBO-FRDO²³ against SIGMAA maps²⁴ were interspersed with XPLOR simulated annealing runs²⁵. Water molecules were added in stereochemically reasonable positions only when equivalent water molecules were observed in all four n.c.s. related regions of SIGMAA difference maps contoured at 2σ . The three water molecules with strongest difference electron density were found to correspond exactly with the three water molecules in 1TRO that bridge repressor to bases A₅, G₆ and A₋₇ (refs 1, 3, 4). These waters and their immediate surroundings were preserved in a simulated annealing omit map²⁶. The final model has one L-tryptophan ligand and 22 water molecules associated with each repressor subunit. The 5' thymidines of the DNA and repressor residues 106–108 could not be identified in electron density maps and are therefore presumed to be disordered. One of several alternative models generated for repressor residues 2–5 had superior stereochemistry and fit to a section of poor but nearly continuous electron density. The R -value calculated with an overall temperature factor (25.4 Å²) is 24.1%; with individual temperature factor refinement this value drops to 21.9%.

* Collected at the Brookhaven National Laboratory Biology Department beamline X12C on a FAST area detector (Enraf-Nonius).

† Collected at the Argonne National Laboratory Beamline X8C on a CCD area detector²⁷.

‡ $R_{\text{sym}} = (\sum_i \sum_j |I(h)_i - \langle I(h) \rangle|) / \sum_i \sum_j I(h)_i$, where h are unique reflection indices.

§ $R = (\sum_h |F_{\text{derivative}} - F_{\text{native}}|) / \sum_h F_{\text{native}}$.

our crystal structure are indirect. The central cavity between repressor reading heads is filled with a hydrogen-bonded water network of $\sim$ two-fold symmetry; water molecules are found at equivalent positions in 1TRO¹ and other repressor structures^{14,15}. The phosphate oxygen facing the major groove side of A/G₋₇ mediates an additional tandem contact between Arg 84c and Asn 87c+.

In 1TRO, the side-chain carboxylate of Asp 46 points towards the minor groove phosphate oxygen of A₁, where it may interact through a bridging water or calcium ion¹. This interaction has been proposed to diminish the affinity of repressor for operator⁴, given that mutation of Asp 46 $\rightarrow$ Asn increases affinity with a 43-bp *trp* operator fragment *in vitro*¹⁶. In our model of the tandem complex, the Asp 46e side chain is in an alternative conformation, rotated away from both the DNA backbone and Lys 90c+ towards a well ordered atom which we have modelled as the α -amino group of the c subunit (Fig. 2a, c). It appears that Asp 46e adopts this conformation to avoid a steric clash with the Lys 90c+ side chain of the tandem dimer (Fig. 2c).

The repressor N-terminal arms contribute to binding affinity without affecting sequence specificity⁵. Our results suggest that the arms enhance the binding affinity of tandemly bound repressors, but probably not a single bound repressor, through two distinct interactions. First, the tandem interaction buries several hydrophobic residues from the solvent. The total burial on each dimer is ~ 480 Å² (of which two-thirds is hydrophobic), a substantial increase over the $\sim 1,430$ Å² buried on each individual dimer upon binding to DNA. Second, the intradimer Asp 46e/ α -Nc interaction stabilizes Asp 46e in a conformation that prevents steric hindrance with Lys 90c+ from the tandem dimer and relieves the poor contact seen in 1TRO between Asp 46 and the DNA phosphate backbone. Because these two interactions are linked through a specific conformation of the arm that depends on the tandem arrangement of repressor

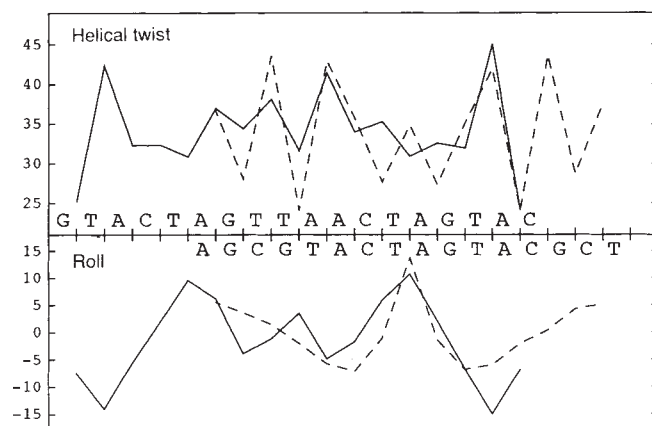


FIG. 3 Comparison of helical twist and roll values for DNA duplexes complexed with *trp* repressor. Solid lines correspond to the duplex of 1TRO; sequence is indicated just above the central horizontal line. Dashed lines correspond to the tandem duplex, with sequence indicated below the central horizontal line. Helical twist and roll values are plotted in degrees on upper and lower vertical axes, respectively. These and other parameters calculated for the tandem duplex have only modest deviation from ideal B-form DNA²⁸. Average values (extreme values) are: tilt, 0.0° (8.5°); propeller twist, -9.0° (-12.6°); slide, -0.3 Å (-1.43 Å); and rise, 3.31 Å (4.05 Å). The central half-site TA step is the locus of bending of the helix axis towards the major groove, and this step has a high positive roll in both duplexes. Both DNA duplexes show a consistent pattern of alternating over- and underwinding of the helix about the base pairs lying directly underneath the repressor dyad axes, even though the base sequences differ. There is extensive contact by repressor to the DNA backbone in this region. Calculations were done with R. Dickerson's NEWHEL92 program obtained through the Protein Data Bank.

dimers, it is not clear whether the alternative Asp 46 conformation could be stabilized in the absence of tandem binding.

Tandem binding suggests an alternative explanation for the genetic sensitivity^{8,9} of base pairs in positions 2–4 (Fig. 1a). The extensive interface between repressor and the phosphodiester backbone of the consensus operator DNA in ITRO had suggested¹ that repressor might sense these base pairs indirectly, by sequence-dependent structural variation in the DNA backbone. Our results suggest that indirect readout may be secondary to tandem recognition, because bases –4, –3 and 2 can be contacted by a tandemly bound dimer in the same way as the central dimer recognizes bases 5, 6 and –7. In addition, the DNA backbones of ITRO and tandem complex duplexes have nearly identical conformations at equivalent base-pair positions, despite several sequence differences (Fig. 3), suggesting that DNA conformation could also be influenced by repressor binding.

Trp repressor can bind to DNA in either single or tandem modes depending on the DNA sequence context. Minor differences at the DNA-binding interface arise from tandem interactions between adjacent dimers, and not from fundamental differences in the mechanism of DNA recognition. The end-on approach of helix E, although limiting the role of the short side chains of the helix N terminus in base-pair recognition, is necessary for sharing of the half-site major groove by parallel E-helices of tandem dimers. An important open question is which mode is used on *trp* operators *in vivo*. Natural half-sites vary slightly in sequence, suggesting a need for adaptability in recognition; flexibility of the repressor reading head¹⁵ and water-mediated hydrogen bonding at the protein/DNA interface^{1,3,4} may be important in achieving this adaptability. Tandem binding could also be a component of regulation by *trp* repressor¹³; its influence on transcription deserves attention. □

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Abbreviations, symbols, units and Greek letters should be identified the first time they are used. Acronyms should be avoided whenever possible and, if used, defined. Footnotes are not used in the text.

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New ideas for personal library maintenance software

Herwig Leirs and Luc De Bruyn

Rapidly expanding personal libraries benefit greatly from bibliographic software; new programs try to take into account each researcher's individual needs.

THE accessibility of scientific literature remains one of the most important components of efficient information management in science. With improved access, through electronic databases, to the ever increasing amount of new literature there has been an expansion in the size of personal libraries which has created fresh problems: it becomes very difficult for researchers to remember where different pieces of relevant information were read and where these publications are stored in their literature collection. A computerized database system seems to be the obvious solution, provided the software takes into account each researcher's specific requirements.

A few years ago, the programs on the market were rather disappointing and many people spent valuable time in trying to store their literature information in simple databases. Nowadays, a number of good software packages are available and there is no longer a need to waste several months in writing your own. However, based on a careful analysis of researchers' needs, we think there is still plenty of scope for improvements in personal library management systems.

Model situation

We imagine a small- to middle-sized group of 1–10 researchers, with various secretarial and technical assistants, working in the same discipline, though possibly with their own particular interests. Most of these researchers will have their own literature collection but would like a common database (up to about 20,000 titles, mainly articles from scientific journals) so that they can consult each other's libraries. It should be possible to grant easy, independent access to students and visiting scientists. Each member of the research group should be able to consult the literature base at more than one computer, but although there may be a local network, not all personal computers (PCs) are connected to it. Some of the researchers use a laptop computer when travelling or have a PC at home; reading articles and preparing manuscripts is precisely the kind of work they like to take with them.

Necessary features

The following features seem essential to any bibliographic software program. (1) Data input must be possible from the keyboard or

by importing information from commercial electronic literature databases such as Current-Contents-on-Diskette, Medline, etcetera, and reprint and/or library requests need to be generated easily for new references. (2) The duplication of identical references must be prevented and spelling mistakes in, for example, journal names must be avoided. (3) Each reference should be linked to keywords, additional information, comments or even abstracts. (4) Editing should be easy and fast throughout the database. (5) Reference selection needs to be available both manually and automatically and the retrieval of references should be possible using the authors' names, the year of publication, keywords, (parts of) words from the title, the journal name, abstract or comments. (6) Selections should be possible using AND, OR, NOT, > and <, or combinations of these. (7) Output formats need to be flexible, ranging from quick listings to sophisticated user-defined "journal format" files (with, for example, a choice between abbreviated or full journal names) which can be used in a word processor. (8) Making manuscript reference lists should, as much as possible, be automated. (9) The user-interface should be simple and unambiguous, preferably menu-driven and using a traditional filing box style; special codes or symbols should be avoided; and a manual must be available. (10) The data should

preferably be stored in a format that can be easily used in a generic database program.

From a recent review of 12 commercially available programs¹, we found that three of them — Papyrus (Research Software Design, Portland, Oregon), Pro-Cite (Personal Bibliographic Software, Inc., Ann Arbor, Michigan) and Reference Manager (Research Information Systems, Inc., Carlsbad, California) — largely fulfilled the requirements listed above. Three others also scored well but we found them to have deficiencies in some areas: EndNote Plus (Niles & Associates, Inc., Berkeley, California) did not support keywords, Notebook II Plus Citation (Pro/Tem Software, Inc., Palo Alto, California) was rather difficult to use and RefMenu (RefMenu, El Cerrito, California) had limited selection operators. Particularly, speed and flexibility scored very well in most of these programs. However, all were found to be rather traditional in terms of keywords (see below) and none had special features to integrate the libraries of researcher colleagues.

We developed MAINBIB² (for DOS-computers, 640 KB RAM and hard disk are needed) which, apart from meeting the other criteria, offers a new approach in these two areas.

Integrating multiple users

Most researchers have a small library of their own; information about their colleagues' libraries is relevant if these colleagues are actually working in the same field. Grouping library information from too many people (such as a whole department or institute) yields too much information of which large parts are of no use to each individual researcher. For this reason it is not imperative for personal library management systems to have huge data storage capacities; instead, they should concentrate on providing all researchers with the freedom they need to run their library in their own way, yet still grant easy access to their colleagues' libraries and vice versa.

A common literature database for several members of the same research group is useful only if it can be accessed simultaneously by each researcher on different computers. Networking provides one solution to this

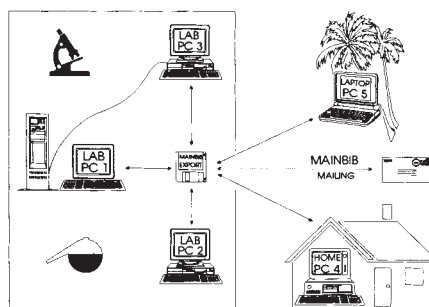


FIG. 1 In order to keep literature information complete and identical on all PCs in one research group (even the ones not used in the office), MAINBIB keeps track of all changes on any PC, exports the updated information to a single floppy disk and imports it to every other PC. Files with references can also be mailed to other colleagues. Duplication of data is avoided during these processes.

The screenshot displays the MAINBIB program interface. At the top, a filecard is shown with fields for AUTHOR (Elisson, G.T.H.), OTHERS (Bronner, G.N. & Taylor, P.J.), YEAR (1993), TITLE (Is the annual cycle in body weight of pouched mice *Saccostomus campestris* the result of seasonal changes in adult size or population structure?), and REFERENCE (J. Zool., London, vol. 229, pp. 545-561, 1979). Below the filecard, a section titled 'MAJOR KEYWORDS' lists various categories. A sub-window titled 'PHYSIOLOGY' is open, showing a list of sub-keywords. Another sub-window titled 'RODENTIA' is also open, displaying a list of rodent species names. The interface uses a text-based menu system with asterisks for selection.

FIG. 2 The basic information filecard in MAINBIB shows a list of major keywords; for each chosen major keyword, a list of sub-keywords to choose from is then displayed. Choosing a keyword is done by tick-marking it with an asterisk. The displayed example is the keyword structure for a research group that is particularly interested in the ecology of African rodents but with only a limited interest in physiology (hence the extensive list with rodent names and the limited number of very general physiological keywords).

problem, provided that all the PCs are connected (which probably won't be the case for computers used at home or laptops). Under these circumstances, the best one can often achieve is to make back-ups from a central computer at regular intervals and distribute these to other machines. Not only is this very time-consuming, but it also restricts the input and editing of data to the single central computer.

With MAINBIB, the program itself keeps track of any changes that occur in the database on every PC and enables these changes to be exported to a single diskette (see Fig. 1). Internal bookkeeping ensures that no duplication occurs and that only the most recent changes are accepted when they are imported into any of the other computers. The whole literature base can thus be used, edited and updated simultaneously on several different computers and, even though there is no physical link, all the computers will ultimately contain the same information. In a similar way this procedure can be used to exchange literature databases with any other research groups that use the same software.

Standardizing keywords

The most common way to select literature is to use keywords that refer to the contents of the article. Sources for these include words from the title or the abstract and keywords provided by the author or journal. Often such keywords are either too general for the specialist or too specialized for the layman. Therefore, researchers attach their own keywords to publications in their library.

Traditionally, every reference was linked to a data field where the user could type a limited number of own (coded) keywords, a technique that requires a lot of disk memory and often results in slow selection procedures. The possibility of spelling mistakes hampered later retrieval. Modern programs now index keywords in lists from which they can be chosen for quick retrieval; one disadvantage is that regular reindexing is needed which is sometimes a slow process. However, there also remains a conceptual problem.

Occasionally, different synonyms or spellings exist for the same keyword which impairs uniformity. Global editing (*a posteriori* making keywords uniform throughout the database) is a remedy but does not prevent problems. A thesaurus-like list of cross-referenced synonyms would be an interesting solution but that possibility is still not well developed. Secondly, when different researchers of the same group, but with slightly different interests, use the same library system, they will tend to use a slightly different set of keywords, for example an ecologist studying dispersal in rodents might use separate keywords for several mice species but not differentiate between insect species, while the entomologist next door studying the same phenomenon with an insect is likely to distinguish between several species of, for example, flies. Similarly, one's interest might change during the years and, gradually, without noticing, one might use more specific keywords in one area and more general ones in another field. Lists of accepted keywords that everybody should follow are an obvious solution. In older

programs, such lists are not foreseen and must be made available in printed form next to each computer.

Where such lists are shown by the program, they are sorted alphabetically. This still does not guarantee that a user will have seen all related keywords before introducing a new one or when formulating a specific search profile. Therefore, MAINBIB uses a rather rigid system of up to 2,500 user-defined keywords that are of interest to the members of the research team. These keywords are grouped per topic and accessible through a limited number of hierarchical levels in order to avoid confusion. Attaching keywords to a reference is done by marking them on a list so that every user is forced to use the same set of possible keywords and spelling mistakes are avoided (see Fig. 2). Obviously, it remains possible to alter or extend such a keyword structure when needed, but this should always remain a very conscious and well-considered process, not routinely done while adding references to the literature database. Due to the pre-set keyword structure, disk space requirements are limited and selection procedures can be accelerated by index-based algorithms instead of character string operations.

The way to go

If personal library bibliographic programs will continue to focus, as they do, on researchers' particular needs, they will further help to increase the efficient use of published information. One area where improvements are still to be expected is the automated production of reference lists for a manuscript. Most procedures that exist so far require the use of special codes in writing the manuscript. Programs like EndNote Plus, Papyrus and Reference Manager already use memory-resident procedures that facilitate pasting such codes into the manuscript. In the near future, new methods will undoubtedly be developed to make use of the multitasking facilities as provided in, for example, Windows. We plan to incorporate this in MAINBIB as well. □

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ERRATUM

A product review, reader service 106, in the 19 August issue (*Nature* **364**; 1993) gave the impression that the SpinBind mini-prep system can be obtained from the FMC Lithium Division. It is, however, available from FMC BioProducts.

Bits and bytes

Event-recording software, a molecular modelling package, graphics toolkits, image processing and analysis packages, and a collection of high-tech hardware options are featured.

NOLDUS Information Technology has released version 3.0 of The Observer, a data collection and analysis **software program for observational research** (*Reader Service No. 101*). The Observer 2.0, which was introduced in 1990, has become a useful tool for behavioural research in pharmacology, psychology, zoology and entomology; version 3.0 should find even broader applications. The Observer now supports all commonly used sampling and recording methods, and coding systems can now have practically unlimited numbers of elements. During an event-recording session, key presses are used to log events, the duration of behavioural states, as well as the time at which these occur. Any data-entry errors can now be corrected online or after the session has been completed. The optional video interfacing module is designed to facilitate coding behaviour from videotape. Connected with a video timecode reader, the software allows event timing independent of the video playback speed. The video recorder can also be controlled from the PC keyboard. Data analysis features have been expanded with reliability analysis, lag sequential analysis, and integrated analysis of behaviour, physiology and environmental parameters. The range of descriptive statistics has been increased, while the analysis of concurrence

puters, observational research kits and an add-on video interfacing module. Versions for Windows and Macintosh computers are under development for release next year.

Shandon has adopted an interactive approach to training cytologists with its **Academy cytology software program** (*Reader Service No. 102*). The course, cervical cytology, comprises 14 modules of 15–20 min duration. Academy uses a desktop system that operates at high speed with high capac-

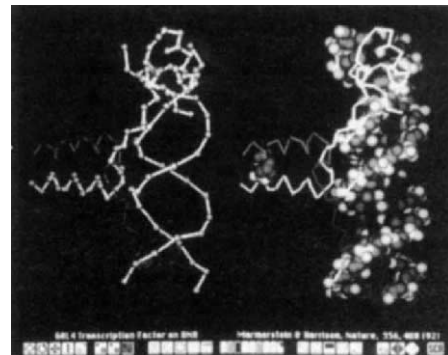


Cytologists can brush up on their technique with Academy software.

ity hard disk storage and high resolution graphics. Computer-aided learning allows the user to zoom in on high-resolution specimen images when required and to repeat modules that need further analysis. It can also act as a refresher training course.

■ Modelling

Molecular Applications Group, a company that develops software for three-dimensional scientific visualization, macromolecular simulation and design, is now shipping the latest version of its **molecular modelling software** for the Apple Macintosh called MacImdad (*Reader Service No. 103*). Developed by Professor Michael Levitt of Stanford University MacImdad is designed to enable a broad cross-section of the scientific community to make use of structural information. "The low cost and ubiquitous nature of the Macintosh platform enables any scientist from principal investigator to lowly undergraduate to view and analyse macromolecular structure", says Andrew Feldman, vice-president of sales and marketing at MAG. The program includes a database that contains all known protein and



Gal 4 transcription factor on DNA.

nucleic acid structures (Brookhaven Protein Data Bank, PDB). The intuitive user interface has been enhanced: uncluttered pull-down menus, dialogue boxes and an icon banner are designed to ensure that powerful features remain easy to use. With the click of the mouse, users can view biologically relevant portions of proteins and nucleic acids by showing folds, bonds, space-filling atoms, Van der Waals' dot surfaces and hydrogen bonds. By pressing the relevant button on the button banner, users can rotate, translate, zoom, superimpose or animate a molecule. MacImdad prints drawings directly to any PostScript printer. Drawings can be saved in black and white or colour in three common formats: PICT, PostScript and screen dumps. These images can be used to create line graphics or colour slides for presentations and publications. The program requires a Mac II or better computer, System 6 or higher, 4 MB RAM and a math co-processor. The list price is US\$2,950; academic discounts and site licences are available.

CADEMO is SciTech's DOS-based program for **computer-aided design of experiments and modelling** (*Reader Service No. 104*). This interactive program helps users formulate experiments, select a statistical model that will accurately reflect the results and perform statistical calculations — all in a way that minimizes experimentation. CADEMO contains specialized expert system modules for designing genetic, chemical, pharmaceutical and industrial experiments and tests. The price of CADEMO for academics is US\$495. Academic site licenses are available.

■ Statistics and graphics

Visual Numerics has launched an X-Windows **graphics toolkit**, known as Exponent Graphics for X, which is available for Sun, Hewlett Packard, DEC, IBM and Silicon Graphics UNIX workstations (*Reader Service No. 105*). The new system includes



The Observer event-recording software can be used for behavioural studies.

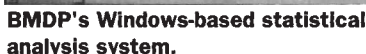
has been further refined. Also new to the program are exploratory graphics, including time-event plots and graphs of continuous signals against observed events. A report generator has been added, and export options to all major spreadsheet, database and statistics packages are provided. The new product line includes a base package for DOS, support packages for handheld com-

In August, Jandel Scientific began shipments of SigmaPlot for Windows, a new **scientific graphing software package** that allows users to produce customized technical graphs that are suitable for publication (*Reader Service No. 106*). Automatic error bars, huge data set handling, nonlinear curve fitting, axis breaks, multiple axes, regression lines, confidence intervals, reference lines are just a few of the program's features.



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BMDP New System (Version 1.0) from BMDP Statistical Software is a complete **statistical analysis system** that takes advantage of the Microsoft Windows user interface (*Reader Service No. 107*). It features



■ Databases

InfoChem, a subsidiary of Springer-Verlag, is offering a **reaction database for the PC** called ChemSelect (*Reader Service No. 108*). First launched in May, ChemSelect offers almost 10,000 core organic and organometallic reactions. It is derived from a database of 1.8 million reactions collected by VINITI from over 1,000 worldwide organic chemical journals from 1975–1988. Chem-

Synopsys Scientific Systems, a supplier of computerized scientific information products and services, announces the release of a new **chemical reaction database**, called Methods in Organic Synthesis (MOS), which is produced in conjunction with the Royal Society of Chemistry (*Reader Service No. 109*). The MOS database is designed to keep chemists up to date with important, recent developments in organic synthesis. It contains about 3,500 new reactions per year and covers topics such as functional group changes, carbon-carbon bond forming reactions, new reagents and synthons, enzymic and biological transformations and the introduction of chiral centres. Data are abstracted from over 90 chemistry journals published worldwide. The first release of the database includes data from the 1993 MOS publications and is initially available for use with the REACCS, ORAC, ISIS and Chem X reaction retrieval systems.

PeakNet is the new **chromatography workstation** from Dionex that provides single-point, PC-based control of the company's DX500 chromatography systems for ion chromatography and HPLC (*Reader Service No. 110*). It includes all the tools necessary to control and automate all aspects of



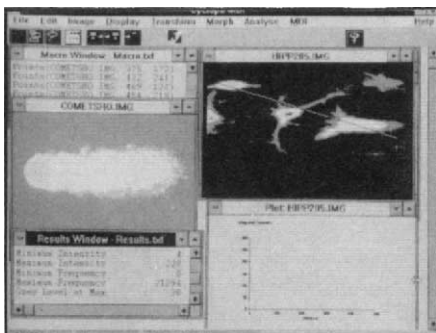
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ules through a high-speed, bi-directional PC-to-module interface called DX LAN. DX LAN sends digital data directly from the modules to the host PC, thereby eliminating the need for external digital conversion devices and reducing signal noise at distances up to 180 m.

FPLCassistant and FPLCdirector are two **chromatography software packages** from Pharmacia Biotech designed to provide advice, knowledge and system control in chromatography (*Reader Service No. 111*). Intended primarily for use with FPLCdirector, FPLC assistant provides support for fast generation of methods, buffer recipes, purification strategies and diagnosis of separation problems. It can, however, be used alone as an independent information system for protein separation techniques, media and columns. FPLCdirector has full system control, data handling and reporting facilities for users of FPLC systems. It is said to expand the potential of FPLC, providing simplified system operation and control, automatic storage of methods and results, and full data manipulation and reporting facilities.

■ Image analysis

Confocal Technologies is now shipping an upgraded version 2.0 of its Cyclops **image processing and analysis software** (*Reader Service No. 112*). An advanced single-screen system, it runs on IBM PCs or compatible computers and PS/2s under Windows 3.1. Enhanced features include full Windows clipboard support and a choice of interactive measurement functions. A range of image import and export facilities allows images

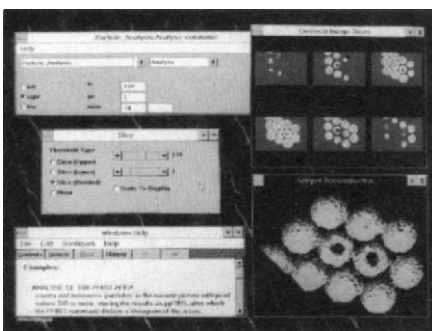


See Cyclops 2.0 for image processing and analysis.

from any digital source to be processed and the software now supports TIFF, TARGA and image file formats from several proprietary instruments. The user interface has been redesigned and now includes toolbars for convenient and rapid access to common functions, and a 'configuration utility', which allows many of the software features to be easily pre-set for noninteractive use. The multiple document interface is designed to offer the flexibility of a completely configurable workspace with multiple image windows, a macro window, extensive plot-

ting, image statistics, spreadsheet output and datalogging, and full text reporting. A basic-like macro language further extends the flexibility. Cyclops 2.0 also supports dynamic data exchange (DDE) and can now be operated within packages such as Microsoft Excel using standard DDE commands.

Semper for Windows is the new **image processing package** from Synoptics that provides users with access to over 1,000 functions covering image analysis and morphology, image processing and enhancement, and picture management and display (*Reader Service No. 113*). The new system provides all of the facilities of the Semper image processing language, yet is designed to allow the user to build solutions to imaging problems quickly and easily by linking



Semper for Windows — see Synoptics.

the appropriate functions together. Solutions can be readily tested and modified as required using the interactive format of the software. Images can be displayed directly on the computer monitor providing a single screen imaging system; multiple images can be viewed simultaneously to allow the effect of the processing routines to be followed and compared to the original image. A menu system provides access to all the Semper commands, which are grouped together according to their function. A system of 'Cue Cards' has been incorporated. Imaging applications can be built by entering Semper commands into a dedicated window directly from the Cue Cards using cut-and-paste techniques or from the keyboard. Each Cue Card is automatically linked to the help facility for that particular command ensuring that a single mouse click provides access to assistance and explanation. Direct control of framestore boards is provided, which enables images to be grabbed from a video input and put directly into a Semper display window. Alternatively, images may be imported in a range of image formats including TIFF and Windows bitmaps.

Phoretix 2D, a follow-up program to the two-dimensional gel electrophoresis analysis package from the company by the same name, is an **image analysis package** that allows densitometry, molecular weight determination and lane comparison analysis for electrophoresis gels (*Reader Service No. 114*). This Windows-based program for the

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PC is capable of accepting images from a wide variety of sources in 8-bit, 12-bit or 16-bit formats. So far, Phoretix says that the software has been configured to accept images from a range of laser densitometers, radiation and fluorescence detection systems, video camera systems and document scanners. At present, up to ten gels and 64 lanes on each gel may be analysed in a single experiment. Following background subtraction, automatic peak identification and peak edge selection, each band may be measured for volume, peak height, molecular weight, R_f value, area and a variety of other values.

Using its newly developed computer program (OligoProbe Design Station), Hitachi Chemical Research Center is now offering an **oligonucleotide probe and primer design service** that should be of interest to researchers performing hybridizations, PCR amplification and antisense drug development (*Reader Service No. 115*). Although some commercially available software packages provide some functions for designing probes and primers, the company says that currently these packages are not able to estimate the specificity of the designed oligonucleotides because there is no global cross-hybridization analysis between probes/primers and existing sequence databases such as GenBank. Therefore, it is still difficult, even for the expert, to design the probe sequences that show the highest specificity with the minimum chance of cross-hybridization to other unrelated genes in the target tissues and cells, without actual hybridization experiments. The OligoProbe Design Station program can extract potential probe sequences from every location of the target gene of the researcher's interest based on a melting temperature (T_m) of probes specified by the researcher. If the target gene is 1,000 base pairs, for example, the computer extracts approximately 1,000 candidate probes, one starting from each nucleotide base in the target gene. Then each candidate probe is analysed for potential hybridization against user-defined databases, typically GenBank, using a proprietary algorithm. The name of genes that cross-hybridize, the position and the estimated T_m of each gene, the location of mismatched nucleotides, the length, content of each nucleotide, hairpin energy and stacking energy for each candidate probe are presented.

■ Computing hardware

Psion's new Series 3a **handheld computer**, which was launched in September, is pocket sized (measuring $6.5 \times 3.3 \times 0.9$ inches) and is said to run for months on two AA-size batteries (*Reader Service No. 116*). Priced at UK£269.95 (256 K model) and UK£329.95 (512 K model), this pocket-sized computer features a large screen (480×160 dots), has voice and sound capabilities, and has built-in database, time management, word-processing and spreadsheet facilities. The large LCD screen accommodates a full-page



Psion's pocket-sized Series 3a computer.

width or text and the software applications are of the same scale and level of sophistication as those found on desktop computers. The Series 3a has a screen that presents 80 characters by 17 lines and can be plugged into desktop computers to share documents, worksheets and other files.

Developed in conjunction with the Advanced Research Project Agency and in only 26 months, the new Cray T3D scalable heterogeneous **supercomputing system** couples proven parallel vector capabilities with massively parallel processing (MPP) capabilities (heterogeneity), enabling users, says Cray, to tackle a wider range of problems than current MPP products are able to do (*Reader Service No. 117*). The Cray T3D is offered in a wide variety of sizes, from a 32-processor version (4.8 peak gigaflops — 4.8 billion floating point operations per second) priced from US\$2.2 million in the United States, on up in powers of two (64, 128, 256, 512 processors) to a 1,024 processor version (153.6 peak gigaflops) with US pricing from US\$31 million. For those with even more to spend — Cray offers a top-of-the-line 2,048 processor version. The system also comes in a wide variety of configurations to allow customers to mix-and-match the proportions of parallel vector and MPP capabilities to particular processing needs.

IBM has introduced major enhancements to its RISC System/6000 family of **workstations and servers**, including new desktop systems that are designed to increase productivity and new high-end models (*Reader Service No. 118*). Four new desktop workstations and server models are offered based on the PowerPC 601 microprocessor, the first in a new generation of computer chips to be developed under IBM's alliance with Motorola and Apple Computer. New graphics adapters are designed to complement the PowerPC technology. Three new high-end models use IBM's own new multi-chip microprocessor, the POWER2. All the new models will run unchanged the existing base of more than 6,500 applications that support AIX/6000, IBM's open, UNIX-based operating system for the RISC System/6000.

Ciprico has added a new member to its high-performance and large capacity **disk array** family — the Ciprico 6720 (*Reader*

Service No. 119). The 6720 is a five-drive array, offering 2 to 4 GB of storage capacity using industry standard 3.5-inch high-capacity SCSI-2 disk drives. In addition to 'hot-swap' drives, the array also supports hot swap redundant power supplies, providing users with multiple levels of fault tolerance. Ciprico's disk arrays can be tailored to popular operating environments, including Novell-compatible networks, Silicon Graphics workstations and other systems supporting standard SCSI-2. Each disk array includes software drivers, utilities and accessories for a specific operating environment. Other features include a 'cold spare' drive shuttle bay allowing users to have a spare drive standing by in the event of a drive failure. The 6720 is packaged in a tower enclosure and lists at US\$16,495 for 4 GB of storage.

AutoMate Scientific is now shipping EasyCode software for the Macintosh — a package that provides an intuitive **graphic interface for programming solution-switching sequences** with the ValveBank line of solenoid valve controllers (*Reader Service No. 120*). Liquid chromatography, tissue perfusion, and gel/blot washing sample programs are included making these applications simpler to program and modify. The software supports control of valves, sensor inputs, interfacing outputs, and sequence loops in up to 16 simultaneous channels. Design features include multiple experiment windows, zoom and reduce capabilities, and a standard cut-and-paste interface. Programmed sequences are downloaded through an included serial cable into AutoMate's ValveBank controller for standalone execution. Programs can also be received by EasyCode from the ValveBank for archiving, further modification or printing.

The 17-inch F560iW Flexscan **high-resolution monitor** from Nanao is similar to its sibling, the 17-inch F550iW, but now allows users to obtain higher resolution at a finer dot pitch (*Reader Service No. 121*). The monitor was developed to meet the needs of users seeking optimum Windows performance and detailed graphics in desktop publishing applications. With a 30 to 82 kHz horizontal and 55 to 90 Hz vertical scanning frequencies, the F560iW can display non-interlaced images at resolutions of $1,280 \times 1,024$ at a 76 Hz refresh rate. The monitor has a 0.26-mm dot pitch Invar Shadow Mask CRT to ensure better focus and sharpness across the increased display area. Other features include ErgoCoat anti-reflective coating, WideView, and dynamic beam spot control and power-saving systems. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal.